Reduction of nitrogen losses by manipulating carbon inputs and pasture composition

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A thesis

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by

William Duncan Talbot

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- Talbot WD, Cameron KC, Di HJ, Malcolm BJ, Whitehead D 2019. Effects of adding readily available carbon to soil on nitrogen losses from cattle urine patches. *New Zealand Journal of Agricultural Research*: 1-22.
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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

Reduction of nitrogen losses by manipulating carbon inputs and pasture composition

by

William Duncan Talbot

The nitrogen (N) cycle is very important to New Zealand agriculture, with N being a key nutrient needed for plant growth. However, there are major environmental concerns relating to the loss of N from agricultural systems. These include nitrate (NO_3^-) leaching and the emission of nitrous oxide (N_2O). Nitrate leaching is of concern due to its potential human health risks and eutrophication of fresh water ways; while N_2O is a potent greenhouse gas and an ozone depleting substance. There is also a significant economic loss to farmers, as lost N needs to be replaced through fertilisers. Pastoral grazing systems have a high risk of NO_3^- leaching and N_2O emissions due to the high N loading rates of urine patches (approximately 613 kg N ha^{-1} for cattle). Carbon (C) has many important interactions with the N cycle, these include immobilisation/mineralisation, nitrification, and denitrification. These interactions are of importance due to their potential to be manipulated in a way that reduces N losses from agricultural systems. Carbon inputs can be manipulated through growing different pasture/crop types with contrasting traits (rooting depth, carbon allocation, root exudates, winter growth), adding urine from animals fed on different diets and applying artificial C inputs. There is however, a lack of detailed knowledge on how C affects the N cycle in New Zealand grazed pastoral systems, on shallow stony soils. The aim of this research was to improve our understanding of these interactions and help develop techniques where C inputs can be manipulated to reduce N losses. To do this three lysimeter trials were conducted at Lincoln University's Ashley Dene Research Development Station (ADRDS). These lysimeters contained Balmoral stony silt loam and were treated with cattle urine.

Lysimeter experiment 1 was split into two trials; the objective of Trial 1 was to determine the effect of artificial inputs of readily available C on the N cycle; the objective of Trial 2 was to determine the effect of different urine compositions and crop types on the N cycle. Trial 1 discovered that applying readily available C to soil significantly reduced N leaching losses, without causing an increase in N_2O emissions. This was attributed to the added C increasing immobilisation of N in the soil. Trial 1 also revealed that the perennial ryegrass (*Lolium perenne*)/white clover (*Trifolium repens*) (PRG/WC) pasture leached

58% less N than the lucerne (*Medicago sativa*) crop. This reduction was attributed to the higher winter plant growth and N uptake of the PRG/WC pasture, reducing the amount of mineral N available to be leached.

Trial 2 discovered that under PRG/WC pasture, urine from cows feed a diet of fodder beet (FB) (*Beta vulgaris*) leached 64% less NO_3^--N than urine from cows feed a diet of PRG/WC; even at the same urine N loading rate. Soil under the FB urine had significantly lower ammonia oxidising bacteria (AOB) *amoA* gene abundance (P = 0.005) and amount of soil NO_3^--N (P = 0.026), suggesting that the FB urine has a biological nitrification inhibitor (BNI) effect. Trial 2 also discovered that the PRG/WC pasture leached 65-84% less N than the bare fallow FB soil. This reduction was attributed to the higher winter plant growth and N uptake of the PRG/WC pasture, reducing the amount of mineral N available to be leached.

Lysimeter experiment 2 discovered that by lowering the urine-N loading rate by 28%, from 700 kg N ha^{-1} to 500 kg N ha^{-1}, N_2O emissions were reduced by 38%, and total N leaching losses were reduced by 39%. This demonstrates that farm management practices that reduce urine-N rate could be an effective way of reducing N losses from grazing systems.

Lysimeter experiment 3 discovered that Italian ryegrass (*Lolium multiforum*)/white clover/plantain (*Plantago lanceolate*) (IRG/WC/P) and perennial ryegrass/white clover/plantain (PRG/WC/P) significantly reduced N leaching losses by 24% and 14%, respectively, compared with traditional PRG/WC. The reasons for these reductions were: (i) the higher plant N uptake, which decreased the soil mineral N content and subsequently reduced the amount of N available to be leached and; (ii) the greater herbage yield which increased transpiration, thus reducing drainage volume.

This research programme has highlighted the importance of manipulating C inputs through winter plant growth and the manipulation of cattle diet in reducing N losses. Increasing plant N uptake over cooler months is potentially an economically viable and effective way of reducing farm N losses. This can be achieved by sowing more winter active crops/pastures, reducing time cattle spend on bare fallow soil and/or through the use of effective catch crops. Manipulating cattle diet by feeding stock low N feed and subsequently reducing the urine N loading rate is potentially an economically viable and effective way of reducing farm N leaching losses; especially if that low N feed such as fodder beet, can provide a BNI effect in the cattle urine.

Keywords: nitrate, ammonium, leaching, nitrous oxide emissions, winter growth, cattle diet, urine, perennial ryegrass (*Lolium perenne*), white clover (*Trifolium repens*), lucerne (*Medicago sativa*), fodder beet (*Beta vulgaris*), Italian ryegrass (*Lolium multiforum*), plantain (*Plantago lanceolate*), ^{15}N isotope, ammonia-oxidising bacteria, grazed forages, herbage N uptake.

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Publications, resulting from this research:

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Publications under review, resulting from this research:

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  (Chapter 6)

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Acronyms and Abbreviations

Ammonia (NH_3)

Ammonia-oxidising archaea (AOA)

Ammonia-oxidising bacteria (AOB)

Ammonium (NH_4^+)

Analysis of variance (ANOVA)

Ashley Dene Research and Development Station (ADRDS)

Biological nitrification inhibitor (BNI)

Carbon (C)

Carbon dioxide (CO_2)

Condensed tannins (CT)

Crude protein (CP)

Di-nitrogen (N_2)

Diverse pasture (DP)

Fodder beet (FB)

Hippuric acid (HA)

Isotope ratio mass spectrometry (IRMS)

Italian Ryegrass (IRG)

Least significant difference (LSD)

Methane (CH_4^+)

Non-urea N compounds (NUNC)

Nitric oxide (NO)

Nitrite (NO_2^-)

Nitrate (NO_3^-)

Nitrogen (N)

Nitrous oxide (N_2O)

Oxygen (O_2)

Perennial ryegrass (PRG)

Plantain (P)

Polymerase chain reaction (PCR)

Quantitative polymerase chain reaction (qPCR)

Water soluble carbohydrate (WSC)

White clover (WC)

Chapter 1

Introduction

1.1 General Introduction

The nitrogen (N) cycle is very important to New Zealand agriculture, with N being a key nutrient needed for plant growth. However, there are major environmental concerns with the loss of N from agricultural systems. These include nitrate (NO_3^-) leaching and the emission of nitrous oxide (N_2O). Nitrate leaching is important due to its potential health concerns and eutrophication of fresh water ways. Eutrophication is a process by which high nutrient levels cause algal blooms and excessive plant growth which consume oxygen, causing aquatic life to die (Smith & Schindler 2009). This can be unsightly and create problems for freshwater recreational activities. Nitrous oxide is a potent greenhouse gas, with a long term warming potential 265 times that of carbon dioxide (CO_2), and an ozone depleting substance (Ravishankara *et al*. 2009; Myhre *et al*. 2013; Pachauri *et al*. 2014). There is also a significant economic loss, as the lost N needs to be replaced by farmers through the use of fertilisers. Pastoral grazing systems have an high risk of NO_3^- leaching and N_2O emissions due to the high N loading rates of urine patches (approximately 613 kg N ha^{-1} for cattle) (Monaghan *et al*. 2005; Selbie *et al*. 2015). There is also an increasing global population (estimated to be 8.1-10.6 billion people by 2050) and increasing demand for animal-derived protein (Ezeh *et al*. 2012; Boland *et al*. 2013). These factors place more pressure on animal grazing systems to intensify and produce more food, while also reducing their environmental N losses. It is therefore necessary to develop an improved understanding of factors that affect these losses and develop potential strategies to reduce these losses.

Carbon (C) has many important interactions with the N cycle, these include immobilisation/mineralisation, nitrification, and denitrification. These interactions are of importance due to their potential to be manipulated in a way that reduces N losses from agricultural systems. Carbon inputs can be manipulated through growing different pasture/crop types with contrasting traits (rooting depth, carbon allocation, root exudates, winter growth), adding urine from animals fed on different diets and applying artificial C inputs. There is however, a lack of detailed knowledge on how C affects the N cycle in New Zealand grazed pastoral systems, on shallow stony soils. The objective of this study is therefore to improve our understanding of these interactions and help develop techniques where C inputs can be manipulated to reduce N losses.

1.2 Aims and Obectives

The aim of the PhD programme was to increase knowledge and understanding of the effects of manipulating C inputs (different crop types with contrasting traits, adding urine from animals fed on different diets and applying artificial C inputs) and pasture composition on N losses/transformation from urine patches on shallow stony soils.

The research program had the following objectives:
Objective 1: To determine the effect of adding readily available C on N losses (NO_3^- leaching and N_2O emissions) from urine treated shallow stony soil and the effect of the C on the key soil N processes of immobilisation and nitrification (including AOB abundance) in urine patches.

Objective 2: To determine the effect of winter plant growth on N losses from urine-treated shallow stony soil and the effect on the key soil N processes.

Objective 3: To determine the effect of manipulating cattle diet on N losses from urine-treated shallow stony soil and the effect on the key soil N processes.

Objective 4: To determine the effect of winter forage, lucerne and pasture systems on N losses and the effect on key soil N processes (in particular AOB abundance), in shallow stony soils.

Objective 5: To quantify the effect of plantain in pasture and the effect of urine from cows grazing on plantain, on N losses and the effect on key soil N processes, in shallow stony soils.

Objective 6: To quantify the effects of plantain and IRG on N leaching losses from urine-treated shallow stony soil, and the effect on the key soil N processes, over a range of urine application dates.

Chapter 2

Literature review

2.1 Introduction

The purpose of this review was to evaluate and synthesise the literature in order to identify gaps in current knowledge, surrounding interactions between C and the N cycle in agricultural soils.

- Understand the key N cycle processes involved with N losses from agricultural soils and factors that influence them.
- Summarise the main issues associated with N losses and review research previously published on N losses from grazed pastoral systems.
- Understand how C inputs interact with the N cycle and how C inputs can be manipulated to reduce N losses.

The knowledge gaps identified in this literature review were used to formulate the research objectives and hypotheses of this thesis.

2.2 Nitrogen cycle

The N cycle is very important to New Zealand agriculture, with N being a key nutrient needed for plant growth. Non-leguminous plants get their N from the soil, which usually contains between 0.1% and 0.6% N. Soil N is usually present in four major forms: (a) organic matter, (b) soil organisms and microorganisms, (c) ammonium ions held by organic matter or clay minerals, and (d) mineral-N forms in soil solution, such as ammonium (NH_4^+) and NO_3^-. The form of N is very important, with only NO_3^- and NH_4^+ being available for plant uptake. Differences in form also affect the ability of N to transfer into the wider environment. Within the soil N cycle there are many inputs to the soil, such as fertiliser, biological fixation, animal manure and atmospheric returns. There are however, many losses of N from the soil, such as volatilization, plant uptake, denitrification and NO_3^- leaching; as shown in the N cycle in Figure 1.

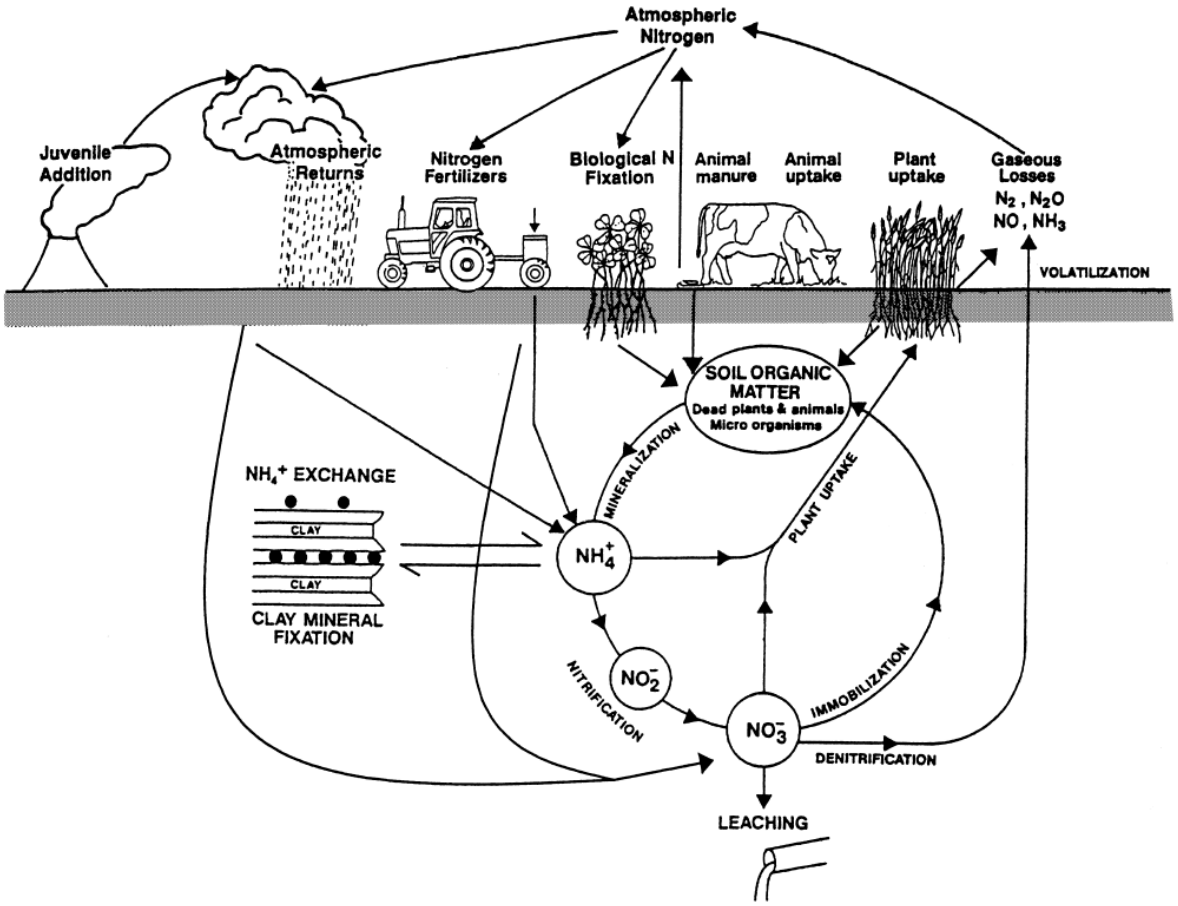

Figure 1. The soil/plant nitrogen cycle (Di & Cameron 2002).

2.3 Processes involved with nitrogen losses

2.3.1 Nitrification

Nitrification is the process in which NH_4^+ in soil is converted into NO_3^-. This reaction is an oxidation reaction which is mainly due to specific nitrifying bacteria. These bacteria are autotrophic (i.e. they get their carbon from CO_2 and their energy from the oxidation of NH_4^+ to nitrite (NO_2^-) and NO_3^-. The reaction is described in the following equations (Kool *et al*. 2007; Cameron *et al*. 2013):

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+ + energy$$

$$2NO_2^- + O_2 \rightarrow 2NO_3^- + energy$$

The two parts of the equation are dependent on different genera of bacteria. The first part of the reaction, where the NH_4^+ is converted to NO_2^-, is mainly conducted by soil ammonia-oxidising bacteria (AOB), such as *Nitrosomonas* and *Nitrosospira* (Cameron *et al*. 2013). These bacteria use the ammonia monoxygenase enzyme to complete this oxidation reaction. This part of the reaction can also be carried out by ammonia-oxidising archaea (AOA). AOA are found in high numbers in soil, however, they do not appear to be as important as AOB in agricultural soil environments such as in high-N dairy

pasture soils (Di *et al*. 2009b). The second part of the reaction, the oxidation of NO_2^- into NO_3^-, is conducted by the genus of bacteria called *Nitrobacter*. This conversion happens very rapidly and therefore NO_2^- rarely accumulates in soil.

The rate that nitrification occurs depends on many soil factors, these include soil moisture, pH, soil temperature, the amount of NH_4^+ present and the concentration of heavy metals. In general nitrification can proceed when there is sufficient water stored for plant growth (Malhi & McGill 1982; Maag & Vinther 1996). However, nitrification rates are optimal around field capacity (~-10 kPa) and generally decline when approaching saturation, as there is little to no oxygen (Malhi & McGill 1982; Maag & Vinther 1996). The rate of nitrification also declines when approaching permanent wilting point (-1500 kPa). The optimal temperature range for nitrification lies between 20°C to 35°C; nitrification is considered to be at a minimum below 5°C and above 40°C (Malhi & McGill 1982; Maag & Vinther 1996; Stark 1996; Andersen & Jensen 2001). An example optimal temperature range for nitrification is shown in Figure 2 (Stark 1996).

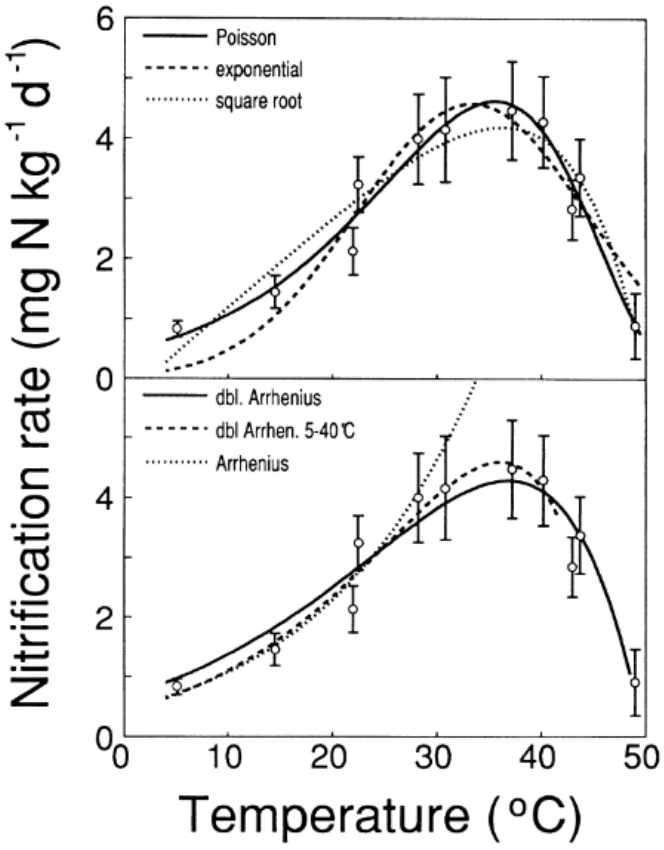

Figure 2. Fit of models to temperature response data for nitrification rates in soils from open grassy interspaces. Data points and error bars represent means and standard errors (*n* = 3) for samples collected from the 0 to 9 cm soil layer of three plots in March (Stark 1996).

Studies have also shown that the optimal soil pH for nitrification generally lies between 4.5 to 7.5 (Haynes 1986; Pietri & Brookes 2008). Increasing soil NH_4^+ also results in higher nitrification rates (Malhi & McGill 1982). While increasing levels of heavy metal concentrations can inhibit nitrification (Cela & Sumner 2002; Rusk *et al*. 2004).

2.3.2 Nitrate leaching

The vast majority of N in the soil is relatively immobile, however, NO_3^- is very mobile. Because most soils are negatively charged, NO_3^- is not held by the soil and is not retained (Di & Cameron 2002). The negative charged NO_3^- ion is repelled by the negatively charged cation exchange sites and is therefore leached from the soil in drainage water. The two main factors affecting the rate of NO_3^- leaching are the concentration of NO_3^- present in soil solution and the amount of drainage that occurs through the soil.

Nitrate is transported as a solute and this is driven by three transport mechanisms. These transport mechanisms are convective transport, diffusive transport and hydrodynamic dispersion.

Convective transport

Convective transport is an important mechanism in NO_3^- movement through soils. Convective transport occurs via the mass flow of water moving through the soil profile. The flow is predominantly vertical, due to gravity, but some horizontal transport can occur. This movement of water, and subsequently NO_3^-, down the soil profile is shown in Figure 3a.

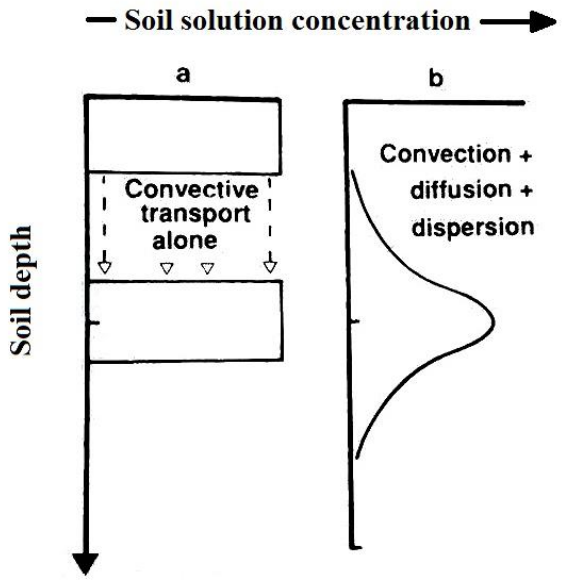

Figure 3. Schematic diagrams of NO_3^- leaching predictions incorporating (a) convective transport alone and (b) convection, diffusion and dispersion. Figure adapted from Cameron and Haynes (1986).

The movement of NO_3^- by convective transport, under saturated conditions, can be described by a modified version of Darcy's law (Hillel 1998; Cameron *et al*. 2013):

$$J_c = qc = -c\left(K\frac{dH}{dx}\right)$$

where J_c is the convective NO_3^- flux, c is the NO_3^- concentration, q is the water flux, K is the hydraulic conductivity and dH/dx is the hydraulic gradient. The hydraulic conductivity (K) is determined by the soil's properties e.g. soil drainage and macropores. The hydraulic gradient is determined by dH/dx, with dH being the height of the soil column plus the water applied. In contrast, dx is the height of the soil column, as shown in Figure 4.

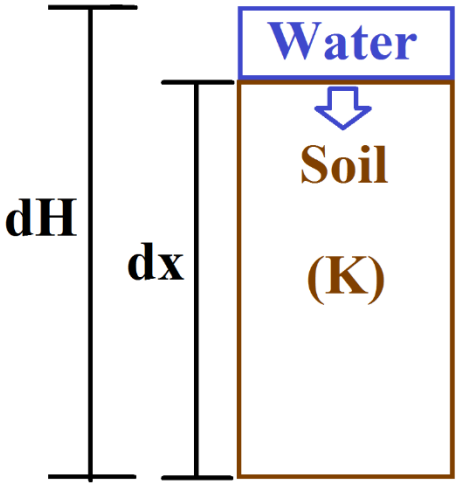

Figure 4. Illustration of how the hydraulic gradient is calculated in Darcy's law.

The distance NO_3^- travels per unit of time by convection depends on the average pore water velocity, U. This can be calculated by using the following equation (Hillel 1998; Cameron *et al*. 2013):

$$U = \frac{q}{\theta}$$

where q is water flux and θ is the volumetric water content. To calculate the pore water flow, Poiseulle's law can be used. This law shows that velocity of the water flow within the soil pore system is highly variable and this is due to differences in soil pore size. This law is described by the following equation (Hillel 1998):

$$Q = \left(\frac{\pi\ p\ g}{8n}\right) r^4$$

where Q is flow rate, p is density, g is acceleration due to gravity, n is viscosity and r is radius of soil pore. The radius is very powerful in the equation as it is raised to the power of four. This means rapid

increases in flow rate with increasing radius of soil pores. Through the use of these equations the convective transport of NO_3^- can be calculated. Convective transport implies a uniform displacement of a band of NO_3^-, however, in reality this band tends to be spread throughout the soil profile, as shown in Figure 3b. This spread is due to diffusive and hydrodynamic dispersion, the two other mechanisms involved in NO_3^- movement through soils.

Diffusive transport

Diffusive transport is the diffusion of NO_3^- from zones of high concentration to zones of low concentration. This mechanism is illustrated in Figure 5, where the NO_3^- molecules will move from the area of high concentration to the area of low concentration, with the concentration eventually evening out over the available area (or volume).

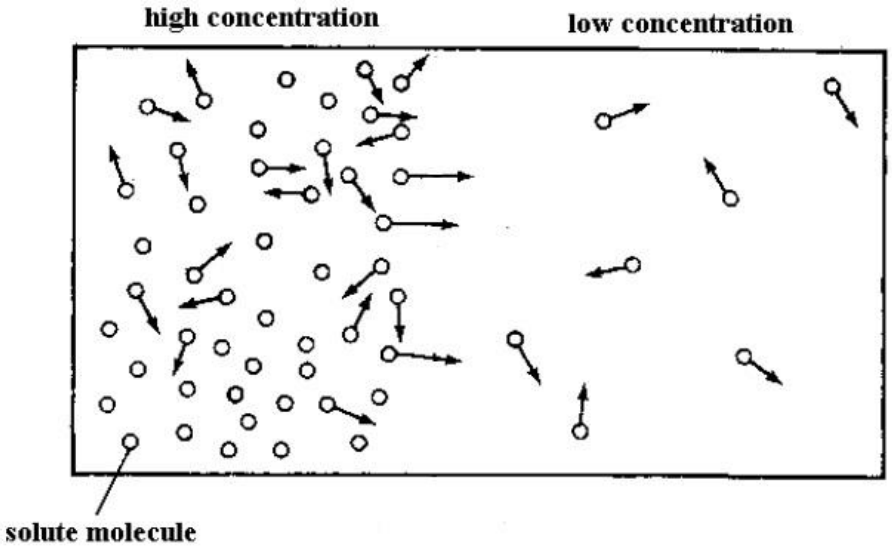

Figure 5. The diffusion of solute molecules from an area of high concentration to low concentration. The solutes will eventually spread themselves equally over the available area (or volume).

The band of NO_3^- flowing through the soil has a higher NO_3^- concentration than in the surrounding soil. This causes diffusion of NO_3^- into the soil around the band. This helps spread the NO_3^- around the peak, as shown in Figure 3b. This rate of diffusion is slower in soils than in pore water, as the flow paths are more tortuous. The diffusion also depends on soil water content, with the rate of diffusion decreasing as the soil becomes drier. The movement by diffusion can be described by Fick's law (Hillel 1998; Cameron *et al*. 2013):

$$J_d = -D_s(\theta)\frac{dc}{dx}$$

where J_d is diffusive flow, D_s is the diffusion coefficient of NO_3^- in soil, θ is the volumetric water content and dc/dx is the NO_3^- concentration gradient.

Hydrodynamic dispersion

Hydrodynamic dispersion is the mechanical action of a solution flowing through soil, causing mixing and spreading. Hydrodynamic dispersion happens due to the large variation in pore size, wide range of pore water velocities (Poiseuile's law), non-uniform flow velocity in a single pore and tortuosity causing a range of flow path lengths, as illustrated in Figure 6. This results in the spreading of the NO_3^- down the soil profile, as shown in Figure 3b. Hydrodynamic dispersion is different to diffusive transport as it does not rely on concentration gradient, but is dependent on the flow of water.

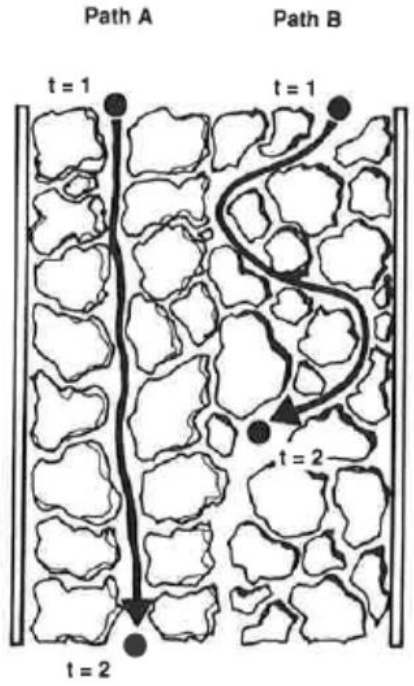

Figure 6. Hydrodynamic dispersion occurring, due to variation in pore size and tortuosity, between two pathways (McLaren & Cameron 1996).

Convective-dispersive equation

The convective-dispersive equation is an equation that combines all three mechanisms (convective, diffusive and dispersive). From this, the total flux of the dissolved NO_3^- can be calculated. The overall equation describing the combined convective-diffusive-dispersive of solute is (Pachepsky *et al*. 2000):

$$\frac{\delta c}{\delta t} = D_a \frac{\delta^2 c}{\delta x^2} - \cup \frac{\delta c}{\delta x}$$

where D_a is the apparent diffusion coefficient and represents the sum of molecular diffusion plus hydrodynamic dispersion, c is the concentration of the solute, t is time, x is the distance, and U is the average pore water velocity.

There are however, complexities that make NO_3^- leaching harder to describe mathematically, these include macropores created by plant roots, earthworms, freezing and thawing cycles and

wetting/drying cracks. Water flow through these macropores can have two effects on leaching: preferential flow and bypass flow. Preferential flow is when the solutes are present in the infiltrating water or when water is applied immediately after a solute. This causes faster leaching than would be expected under convective flow alone. Bypass flow is slower leaching as the solutes present in the soil aggregates are protected from leaching. Biological transformation processes, such as nitrification, denitrification and immobilization, can remove, or add NO_3^- to soil solution. These transformations are extremely difficult to describe mathematically since the transformations occur spasmodically rather than steady state.

2.3.3 Denitrification

Denitrification is the loss of gaseous N from the soil. The main mechanism is through biological denitrification, a less important mechanism is chemical denitrification. Biological denitrification occurs in poorly drained soils where anaerobic conditions occur. The process is carried out by anaerobic bacteria. These bacteria use NO_3^- instead of oxygen (O_2) as an electron acceptor in metabolic reactions. An equation describing the reaction is (Saggar *et al*. 2013):

$$C_6H_{12}O_6 + 4NO_3^- \rightarrow 6CO_2 + 6H_2O + 2N_2 \uparrow$$

The reduction of NO_3^- usually proceeds in a series of steps, with the constant loss of O_2 turning NO_3^-, in turn to NO_2^-, nitric oxide (NO), nitrous oxide (N_2O) and finally di-nitrogen (N_2). This is described in the following equation, along with the enzymes responsible (Kool *et al*. 2007; Saggar *et al*. 2013):

$$2NO_3^- \xrightarrow{NO_3^- reductase} 2NO_2^- \xrightarrow{NO_2^- reductase} 2NO\uparrow \xrightarrow{NO\ reductase} N_2O\uparrow \xrightarrow{N_2O\ reductase} N_2\uparrow$$

The enzymes involved are associated with most nitrifying bacteria, however, some bacteria are only capable of completing part of the reduction (partial denitrifiers). Throughout this conversion, the amount of NO lost is small and occurs only in acidic conditions. However, most of the N_2O produced escapes as a gas, before it can be reduced into N_2, which is also lost as a gas.

2.4 Nitrogen losses

2.4.1 Consequences of nitrogen losses

Nitrogen leaching

Nitrogen leaching is of importance because it can have serious negative environmental, health and economic impacts. When N enters into waterways and ground-water it can cause eutrophication (Smith *et al*. 1999). Eutrophication leads to increased growth of algae and other aquatic plants, which can be unsightly and create problems for freshwater recreational activities. There is also a human health risk from increased NO_3^- concentrations (i.e. >11.3 ppm NO_3^--N) in drinking water (Grizzetti *et*

al. 2011). The ingested NO_3^- is converted into NO_2^- in the stomach and this can be absorbed into the bloodstream reducing the blood's oxygen carrying capacity, which can potentially cause death from cellular anoxia. As soil nitrogen is a key nutrient for plant growth, valuable N lost through leaching must be replaced through fertilisers, adding an extra economic cost for farmers.

Nitrous oxide emissions

Nitrous oxide is a greenhouse gas and an ozone depleting substance. The long term global warming potential of N_2O is 265 times that of CO_2 (Myhre *et al*. 2013; Pachauri *et al*. 2014). It has also been shown that N_2O has become and will remain the biggest threat to the ozone layer in the 21st century (Ravishankara *et al*. 2009). As stated earlier, N is a key nutrient to grow grass on farms. The loss of N as gas through de-nitrification presents an additional economic cost to the farmer, who will need to replace lost N via fertiliser.

2.4.2 Amounts of nitrogen lost from grazed pastoral systems

Grazed pastoral systems, where milk, meat and fibre are produced from ruminants, can vary widely in intensity; ranging from extensive sheep to intensive cattle systems. However, one common feature of these systems is the inefficient use of dietary N. Ruminants only use a small proportion of the N ingested (5-30%), with 70-95% being excreted as urine or dung (Oenema *et al*. 2005; Selbie *et al*. 2015); over 60% of this N is excreted through urine. The urine-N rate under a cattle urine patch can range from 200-2000 kg N ha^{-1}, however, a meta-analysis by Selbie *et al*. (2015) found the average urine-N rate to be 613 kg N ha^{-1} for dairy cattle (grazing a predominately pasture diet). The average cattle urine patch had a urine-N concentration of 6.9 g N L^{-1} (n = 51), average volume of 2.1 L (n = 8), deposited onto an average area of 0.24 m^2 (n = 6) (Selbie *et al*. 2015). Selbie *et al*. (2015) also performed a review on the fate of urine in grazed forage systems, which estimated typical values of urine-N recovery to be: 41% plant uptake, 20% NO_3^- leaching, 26% immobilisation (soil), 2% N_2O emissions, and 13% NH_3 volatilisation. Urine patches from grazed pastoral systems are major sources of N losses. Soil type can also have a large impact on N losses, with shallow stony soils being of particular importance as they are highly vulnerability to N leaching losses, been subject to recent land use intensification and are extensive in Canterbury (Carrick *et al*. 2013; Carrick *et al*. 2014). Crop type and urine application date also have important impacts on N losses from grazed pastoral systems. Examples of NO_3^--N leaching losses and N_2O-N emissions from New Zealand grazed pastoral systems with cattle urine applied have been consolidated into Table 1 and Table 2, respectively.

Table 1. Examples of nitrate-N leaching losses from urine patch areas in common grazed plants. Perennial ryegrass (PRG). White clover (WC). Italian ryegrass (IRG). Fodder beet (FB). Plantain (P). *Values include ammonium leaching losses.

Reference	Soil	Urine application	Crop	Nitrogen applied (kg N ha^{-1})	Nitrate leaching losses (kg NO_3^--N ha^{-1})	(% of N applied)
Cameron *et al*. (2007)	Yellow brown pumice soil (free draining)	Autumn	PRG/WC	700	133-306	19-43
Carey *et al*. (2017)	Balmoral stony silt loam	Winter	Winter grazed kale soil	350	131-167	37-48
				700	193-264	18-38
Carey *et al*. (2016)	Balmoral stony silt loam	Winter	Winter grazed kale soil	350	172-272*	49-78
Carlton *et al*. (2018)	Paparua fine sandy loam	Summer	PRG/WC	700	37-126	5-18
			PRG/WC/P	700	3-41	0.4-6
Di *et al*. (2009a)	Lismore stony silt loam	Autumn	PRG/WC	1000	399	40
	Harihari recent silt loam				68-123	7-12
	Mataura recent sandy loam				436-457	44-46
Di and Cameron (2007)	Lismore stony silt loam	Autumn	PRG/WC	300	60	20
				700	188	27
				1000	255	26
Malcolm *et al*. (2016)	Balmoral/Lismore stony silt loam	Winter	Winter grazed FB soil	250	79	32
				300	64	21
Malcolm *et al*. (2015a)	Balmoral stony silt loam	Winter	Winter grazed kale soil	500	213	43
				700	380	54
Malcolm *et al*. (2014)	Templeton sandy loam	Autumn	PRG/WC	1000	307-416	31-42
			IRG/WC	1000	230-317	23-32
			Tall fescue/WC	1000	448-496	45-50
Maxwell *et al*. (2018)	Templeton sandy loam	Autumn	IRG	700	143	20
			PRG	700	178-267	25-38
Menneer *et al*. (2008)	Kuratau loamy sand	Autumn	PRG/WC	775	114	15
Shepherd *et al*. (2014)	Horotiu silt loam	Autumn	PRG/WC	1000	244-619	24-62
	Waikare clay				153-452	15-45
	Oropi sandy loam				233-396	23-40
Shepherd *et al*. (2010)	Horotiu silt loam	Winter	PRG/WC	500	72	14

Welten *et al*. (2019)	Horotiu silt loam	Summer	PRG	622	40	6
			Plantain	622	34	5
			Lucerne	622	325	53
		Autumn	PRG	622	77	12
			Plantain	622	56	9
			Lucerne	622	203	33
		Winter	PRG	622	117	19
			Plantain	622	58	9
			Lucerne	622	235	38
Welten *et al*. (2013b)	Immature Orthic pumice soil (free draining)	Autumn	PRG/WC	600	217	36
Woods *et al*. (2018)	Templeton sandy loam	Autumn	PRG/WC	664	113*	17
				700	113*	16
			IRG/WC/P	508	13*	25
				700	62*	9
Woods *et al*. (2016)	Templeton sandy loam	Autumn	PRG	700	205*	29
			IRG	700	133*	19
			Lucerne	700	407*	58
Zaman and	Paparua silt loam	Autumn	PRG/WC	600	81	14
Blennerhassett (2010)		Spring	PRG/WC	600	52	9

Table 2. Examples of nitrous oxide emissions from urine patch areas in common grazed plants. Perennial ryegrass (PRG). White clover (WC). Italian Ryegrass (IRG). Fodder beet (FB). Diverse pasture (DP). *Values are based off N_2O-N emissions from only urine-N, as control value N_2O-N emissions were subtracted from the treatments total N_2O-N losses.

Reference	Soil	Urine application	Crop	Nitrogen applied (kg N ha^{-1})	Nitrous Oxide emissions	
					(kg N_2O-N ha^{-1})	(% of N applied)
Di and Cameron (2008)	Templeton fine sandy loam	Autumn	PRG/WC	1000	4.11	0.4
Qiu *et al*. (2010)	Templeton fine sandy loam	Winter	PRG/WC	1000	12.7	1.3
		Summer			7.8	0.8
Di *et al*. (2007)	Taupo pumice sand	Winter	PRG/WC	700	1.0	0.1
	Templeton fine sandy loam	Winter		1000	20.9	2.1
	Lismore stony silt loam	Autumn		1000	8.7	0.9
	Horotiu silt loam	Autumn		1000	6.2	0.6
van der Weerden *et al*. (2011)*	Horotiu silt loam	Autumn	PRG/WC	496	0.5	0.1
		Spring		551	2.2	0.4
	Te Kowhai silt loam	Autumn		496	2.5	0.5
		Spring		551	5.2	0.94
	Ngamoka silt loam	Autumn		504	0.7	0.14
		Spring		548	0.3	0.05
	Wilford hill silt loam	Autumn		504	0.4	0.07
		Spring		548	0.5	0.09
	Wingatui silt loam	Autumn		499	4.5	0.91
		Spring		548	2.2	0.41
	Otokia silt loam	Autumn		499	2.5	0.5
		Spring		548	1.2	0.21
De Klein *et al*. (2011)	Templeton fine sandy loam	Autumn	PRG/WC	1000	12.7-14.4	1.3-1.4
	Pukemutu silt loam	Autumn		610	5.9-9.2	1.0-1.5
Hoogendoorn *et al*. (2008)	Wilford Hill silt loam	Spring	PRG/WC	360	0.31-4.14	0.1-1.2
	Warepa silt loam				0.39-3.16	0.1-0.9

Di *et al*. (2010)	Lismore stony silt loam	Autumn	PRG/WC	1000	31.4-39.8	3.1-4.0
	Harihari recent silt loam				13.9-19.7	1.4-1.9
	Mataura recent sandy loam				16.4-21.3	1.6-2.1
	Horotiu silt loam				16.6	1.7
Luo *et al*. (2010)	Horotiu silt loam	Autumn	PRG/WC	1000	2.9-3.4	0.3
	Oropi sand soil				27.4-35.9	2.7-3.6
	Waikare clay				18.6-23.4	1.9-2.3
Di and Cameron (2012)	Templeton silt loam	Winter	PRG/WC	1000	1.1	0.1
Luo *et al*. (2015a)	Otorohanga silt loam	Autumn	PRG/WC	700	1.6	0.2
		Winter			1.8	0.2
Zaman *et al*. (2013)	Typic Haplustepts silt loam	Autumn	PRG/WC	600	6.3-7.6	1.1-1.3
		Spring			5.0-5.7	0.8-1.0
Selbie *et al*. (2014)	Moorepark sandy loam	Winter	PRG	300	1.7-3.8	0.6-1.3
				500	1.6-3.1	0.3-0.6
				700	3.4-7.8	0.5-1.1
				1000	3.7-7.4	0.4-0.7
Monaghan *et al*. (2013)	Pukemutu silt loam	Winter	Kale	399	7.7	1.9
				528	3.9	0.7
Van der Weerden and Styles (2012)	Te Houka silt loam soil	Winter	Swede	190	8.2-8.9	4.3-4.6
Di *et al*. (2016)	Templeton silt loam	Autumn	IRG	700	9.4	1.3
			Lucerne		7.1	1.0
			PRG/WC		7.6-27.4	1.1-3.9
			DP	500	4.0	0.8
			PRG/WC		3.4	0.7
			DP	700	7.1	1.0
			IRG/Plantain	507	14.5	2.9
				700	33.0	4.7
			PRG/WC	672	20.0	3.0
	Balmoral stony silt loam	Winter	Kale	300	6.2	2.1
			FB		3.9	1.3

Luo *et al*. (2018)	Horotiu silt loam	Summer	Lucerne	622	4.6	0.7
			Plantain		3.3	0.5
			PRG		2.3	0.4
		Autumn	Lucerne		1.2-1.8	0.2-0.3
			Plantain		0.9-1.2	0.1-0.2
			PRG		1.4-1.9	0.2-0.3
		Winter	Lucerne		1.4	0.2
			Plantain		0.8	0.1
			PRG		3.0	0.5
van der Weerden *et al*. (2017)	Koau deep silty clay loam	Winter	Kale	550	2.6-7.4	0.5-1.3

2.5 Interactions within the nitrogen cycle that can affect nitrogen losses

Carbon plays an important role in the N cycle in agriculture. Carbon interacts with the N cycle through mineralisation/immobilisation, nitrification, denitrification and plant N uptake. These interactions are of importance due to their potential to be manipulated in a way that reduces N losses from agricultural systems.

2.5.1 Mineralisation and immobilisation

Mineralisation

Mineralisation is the conversion of organic forms of N into inorganic forms of N or mineral forms. The general term mineralisation refers to a series of reactions, which can be simplified into a few stages. The complex proteins are broken down into amino acids. These amino acids are broken down into ammonia (NH_3). This breakdown is called ammonification and provides microorganisms with energy. An equation describing the reaction is (McLaren & Cameron 1996):

$$\underset{(amino\ N)}{R - NH_2} + H_2O \rightarrow NH_3 + R - OH + energy$$

The NH_3 produced is hydrolysed to NH_4^+. Mineralisation is caused by soil microbes as they need inorganic forms of N for their own use. The microbes convert organic N to inorganic N by excreting exocellular enzymes into the soil. If there is excess mineral N, it is released into the soil and taken up by plants. This release of mineral N has important consequences for plant nutrition.

Immobilisation

Immobilisation is the conversion of inorganic N to organic N. Once the microbes described in the last section take up the mineral N, they convert it in their bodies into organic forms for their own use. When there is a shortage of mineral N, the microbes can take up mineral N which may be available from the surrounding soil (e.g. NH_4^+ or NO_3^- in soil solution), making it unavailable to plants.

Net rates of mineralisation and immobilisation

The mineralisation and immobilisation processes happen at the same time (working in reverse of one another). When the rate of one exceeds the other, a net result occurs e.g. when a higher rate of mineralisation compared to immobilisation occurs, the result is net mineralisation. Whether microbial activity causes net immobilisation or net mineralisation depends on the C:N ratio of the organic matter, the bioavailability of the C and other soil environmental conditions (e.g. soil temperature and soil water).

Net mineralisation (release of inorganic N) generally occurs when the organic material being decomposed has a relatively high N content compared to its C content. These high levels of N give a low C:N ratio. Examples of this are pelletized poultry manure (C:N ratio of 10:1), green waste-based composts (C:N ratio of 16:1), straw based composts (C:N ratio of 11:1) and vermi-cast (C:N ratio of 12:1) all of which resulted in net mineralisation (Flavel & Murphy 2006). Mineralisation happens because there is an excess of inorganic N for the microbes to use, allowing the excess to go into the soil solution.

In contrast, immobilisation generally occurs when there is a low N content, meaning a high C:N ratio. In general, the application of organic materials with a C:N ratio higher than 20-40 promotes net immobilisation (Chaves *et al*. 2005). Immobilisation happens because the low N content, compared to the high C content, means there is a sufficient amount of energy from the carbon, but a lack of N. This lack of N causes microbes to take mineral N from the surrounding soil e.g. NO_3^- and NH_4^+. This results in a net change of inorganic N being converted into organic N. An example of this is barley straw, which has an C:N ratio of 66:1 (Malcolm *et al*. 2019). Examples of approximate C:N ratios of other plant and soil materials are shown in Table 3.

Table 3. Approximate composition of organic C, total N and the C:N ratios of plant and soil materials. Adapted from Follett *et al*. (1981).

Organic material	Organic C (%)	Total N (%)	C:N ratio
Lucerne (young)	40	3	13:1
Clover (mature)	40	2	20:1
Wheat straw	40	0.5	80:1
Soil humus	2	0.2	10:1
Soil bacteria	50	10	5:1
Soil actinomycetes	50	8.5	6:1
Soil fungi	50	5	10:1

The bioavailability of the C is also an important factor for the net rate of immobilisation or mineralisation (Bengtsson *et al*. 2003; Chaves *et al*. 2005; Shepherd *et al*. 2010; Malcolm *et al*. 2019). Chaves *et al*. (2005) found lower immobilisation rates when the C source applied had a higher lignin content (a relatively non-bioavailable form of C) and suggested a good immobiliser must have a high C:N ratio and be easily decomposable (i.e. low lignin content). Bengtsson *et al*. (2003) suggested the ATP content of the C was more important than the C:N ratio in influencing net rate of immobilisation or mineralisation. Shepherd *et al*. (2010) found the addition of sucrose (an extremely bioavailable form of C) significantly increased immobilisation of N. Malcolm *et al*. (2019) found similar results, when barley straw (C:N ratio of 66:1) was incorporated into bare soil after urine deposition, increased immobilisation of N was observed; in contrast the addition of spent woodchip bedding (C:N ratio of

29:1) was shown to have no effect on immobilisation. Malcolm *et al*. (2019) highlighted the importance of the form of C in organic materials in relation to rate of immobilisation or mineralisation and suggest future studies should explore the compositional features of C-rich material (e.g. lignin and cellulose content) that provide the greatest potential for immobilisation.

Artificial C inputs used to stimulate immobilisation/mineralisation

Artificial C inputs (e.g. sawdust, sucrose, glucose) have been used in some studies to increase the rate of immobilisation and subsequently to reduce N leaching. Many studies have shown that incorporating high C inputs into soil can result in significant amounts of N immobilisation and in many cases a reduction in N leaching (Kanal 1995; Sarrantonio 2003; Chaves *et al*. 2005; Chen & Xu 2005; Szili-Kovács *et al*. 2007; Chaves *et al*. 2008; Shepherd *et al*. 2010; Malcolm *et al*. 2019). Shepherd *et al*. (2010) found that when sucrose, a readily available high C input, was applied to cattle urine patches on PRG (*Lolium perenne*)/WC (*Trifolium repens*) at 12 or 24 t ha^{-1}, this reduced N leaching, relative to the control urine patches, by 27% and 66%, respectively. The effect of different C:N ratios of inputs on NO_3^- leaching can be seen in Figure 7. However, increasing immobilisation by using artificial C inputs may not be an efficient way of reducing N losses because the immobilisation of mineral N can lead to a yield decrease and the large C applications required may not be practical or economical (Shepherd *et al*. 2010).

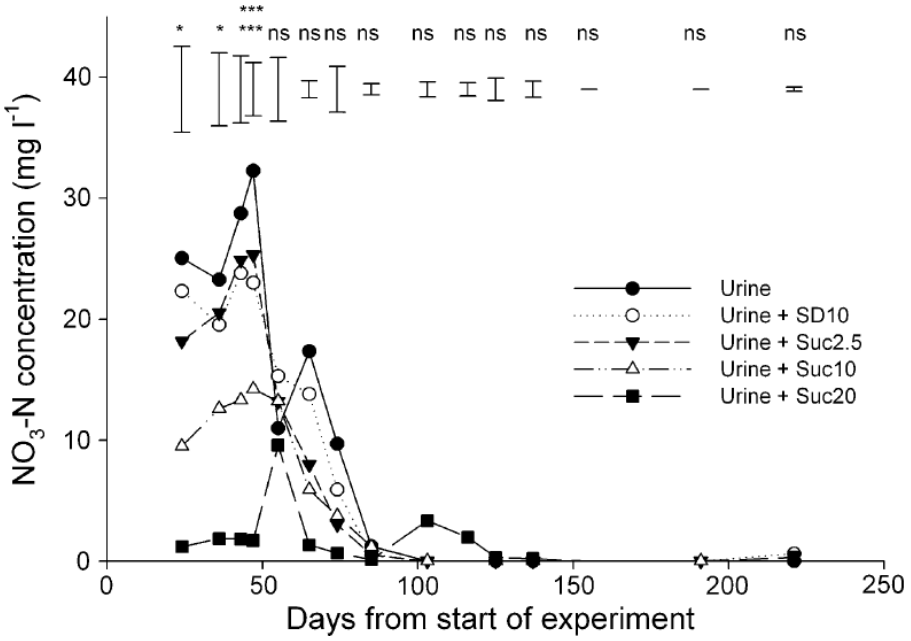

Figure 7. Nitrate (NO_3^--N) concentration profile in leachate draining from lysimeters that received urine and different carbon inputs (Suc = sucrose, SD = sawdust, number represents target C:N ratio of treatment). Bars represent SED; **P* < 0.05, ***P* < 0.01 and ****P* < 0.001, unless designated ns (not significant) (*n* = 5). Levels of significance are for comparison between urine treatments. Note: limited drainage occurred after day 76. Figure adapted from Shepherd *et al*. (2010).

Net mineralisation can be stimulated through the addition of high N containing inputs, such as molasses (C:N ratio of 18-24.2) (Rahn *et al*. 2003; De Neve *et al*. 2004). De Neve *et al*. (2004) found that the addition of compost, with a high C:N ratio of 33.6, caused immobilisation. However, 15 weeks later, when molasses (C:N ratio of 24.2) was applied a significant re-mineralisation occurred (equivalent to 73% of the N initially mobilised).

There is very little information on the effects of organic amendments used to immobilise N from urine patches and no research has focused on immobilisation of deposited urinary N in New Zealand winter forage crop and lucerne systems. This is a significant gap in knowledge and an important area for further investigation.

2.5.2 Denitrification rate

The literature suggests there is a strong relationship between readily available organic C in soil and the denitrification rate (Bremner & Shaw 1958; Burford & Bremner 1975; Weier *et al*. 1993). It is suggested that the addition of C to soil provides the organic C needed by soil denitrifiers and stimulates microbial growth and respiration.

2.5.3 Plant root exudates

Plants release root exudates into the rhizosphere, the narrow zone of soil around the roots. This zone is hugely influenced by the plant and has a wide range of microorganisms and invertebrates (Chen *et al*. 2006; Philippot *et al*. 2013). Root exudates include secretions of ions, enzymes, free oxygen and water, mucilage, and a diverse array of carbon-containing primary and secondary metabolites (Bertin *et al*. 2003; Uren 2007). Plants can release nearly 25% of the C fixed photosynthetically into the rhizosphere (Marschner 1995; Walker *et al*. 2003). These exudates shape the soil bacterial community structure, through positive or negative interactions with soil microbes (el Zahar Haichar *et al*. 2008). These microbes, which include AOAs, AOBs and denitrifiers, can have major effects on the plant and the soil environment around the plant root and play important roles in nitrification, denitrification and subsequently N losses.

An important group of root exudates are biological nitrification inhibitors (BNI). These exudates suppress nitrifying microbes in the rhizosphere, which in turn has a major effect on the N cycle, as it leads to a reduction of nitrification in soil. For example, Subbarao *et al*. (2009), found a 90% decline in soil NH_4^+ oxidation rates and N_2O emissions with the establishment of the BNI producing *Brachiaria humidicola* pastures. The release of BNI from plant roots has been seen in many studies (Subbarao *et al*. 2006a; Subbarao *et al*. 2007b; Subbarao *et al*. 2007a; Subbarao *et al*. 2013). Multiple species have been shown to produce BNIs, including sorghum (*Sorghum bicolor*), a wild relative of wheat (*Leymus*

racemosus), *Brachiaria humidicola* and pearl millet (*Pennisetum glaucum*) (Subbarao *et al*. 2007b). This adaptation is hypothesised to be a mechanism for plants to conserve N and increase their N use efficiency in low-N ecosystems (Subbarao *et al*. 2012). Recent research by Carlton *et al*. (2018) has discovered that the herb plantain (*Plantago lanceolata*) may release a BNI which could help to reduce nitrate leaching in grazed pasture systems.

The inhibition of the conversion of NH_4^+ to NO_3^-, extends the time that NH_4^+ remains in soil and is plant available. This is important as NH_4^+ is much less mobile than NO_3^-, which can easily be lost to the environment through leaching and gas emissions. Most plants and microbes have the ability to utilize NH_4^+ or NO_3^- as their mineral N source (Haynes & Goh 1978). However, plants prefer a mixture of NH_4^+ and NO_3^- for their N source (Michael *et al*. 1970). The assimilation of NO_3^- requires an energy equivalent to 20 moles of ATP $mole^{-1}$ of NO_3^-. In contrast, the assimilation of NH_4^+ requires an energy equivalent to 5 moles of ATP $mole^{-1}$ of NH_4^+ (Salsac *et al*. 1987). These energy savings from the assimilation of NH_4^+ could lead to higher production of biomass (Subbarao *et al*. 2006b). With BNI producing plants, a shift could occur towards NH_4^+ dominated crop nutrition. These low-nitrifying systems could potentially benefit both agriculture and the environment. However, our understanding of BNI function in plants is new and still developing (Subbarao *et al*. 2015; Carlton *et al*. 2018).

Little research has been done on New Zealand winter forage, lucerne and pasture systems in relation to plant exudates and their impact on the N cycle. Further work on plant BNI effects on N losses is urgently needed to reduce environmental impacts of pastoral farming.

2.5.4 Plant nitrogen uptake

Plant uptake of mineral-N can significantly reduce the amount of N available to be leached and thus reduce N losses. However, different plants uptake N at different rates and at different times of the year. As the majority of N leaching occurs in late autumn/winter, high late autumn/winter growth rate has been suggested as a method of reducing N losses.

Welten *et al*. (2019) found that lucerne leached between 205.3 – 362.2 kg N ha^{-1} (because it was inactive in winter), whilst ryegrass leached 40.2 – 140.7 kg N ha^{-1}, when urine (622 kg N ha^{-1}) was applied at various dates. Woods *et al*. (2016) reported similar findings, with PRG/WC leaching 205 kg N ha^{-1}, compared with lucerne leaching 407 kg N ha^{-1}, when urine (700 kg N ha^{-1}) was applied in May. This research shows the potential of reducing N leaching losses through selecting plants with higher winter growth rates.

Italian ryegrass (*Lolium multiforum Lam*.) a winter active ryegrass, has also been proposed as a potential tool for reducing N leaching and N_2O emissions from farms. Italian ryegrass has significantly

greater activity and growth in winter than traditional perennial ryegrass (Kemp 1999; Charlton & Stewart 2000). In a glasshouse study, Moir *et al*. (2013) found that two IRG cultivars 'Feast 2' (134 kg N ha^{-1}) and 'Tama' (130 kg N ha^{-1}) leached less N than two PRG cultivars 'Aber Magic' (280 kg N ha^{-1}) and 'Alto' (310 kg N ha^{-1}), when urine (700 kg N ha^{-1}) was applied. Malcolm *et al*. (2014) used a lysimeter study, to compare N leaching losses of four pasture species compositions over two winters. Urine (1000 kg N ha^{-1}) was applied in May of each winter. Italian ryegrass/white clover leached 25% and 24% less N than the traditional PRG/WC over the two winters. Woods *et al*. (2016) also found in a lysimeter study that pure IRG leached 35% less N than PRG/WC, under a urine patch (700 kg N ha^{-1}) applied in May. Maxwell *et al*. (2018) compared N leaching losses from pastures of four different PRG cultivars ('Tyson', 'Arrow', 'AberDart' and 'One 50') against pure IRG (cultivar 'Tabu'). This study found that IRG leached 33-46% less N than the pure PRG pastures, under a urine patch (700 kg N ha^{-1}) applied in late April. However, there has been no research on the effect of IRG on reducing N losses from shallow stony soils.

Little research has been done on New Zealand winter forage, lucerne and pasture systems in relation to winter plant growth and their impact on the N cycle, in shallow stony soils. Research is needed to investigate and quantify the effect winter plant growth has on the N cycle, in shallow stony soil.

2.5.5 Urine

Urine-Nitrogen

The plant species being grazed by an animal can have a large effect on the form of N and total N found in the animal urine. Generally, higher N intake by an animal leads to higher urinary-N excretion (Haynes & Williams 1993; Selbie 2014). Higher rates of urinary N can result in greater losses of N through NO_3^- leaching and N_2O emissions (Luo *et al*. 2008; Malcolm *et al*. 2015a). Ruminants on pasture commonly ingest excess protein, however, they are energy limited. This leads to higher ruminal ammonia concentrations being excreted in the urine as urea (Whitehead 1995). Animals on a maintenance-only diet require around 7% of their DM intake to be crude protein (CP), pregnant animals require 10-12%, while lactating animals require 15-20% (Thompson & Poppi 1990). High quality pastures can have a CP content of up to 30% (Waghorn *et al*. 2007). Manipulating the water soluble carbohydrate (WSC) and CP composition of plants has been proposed as a potential method to reduce urine N concentration, leading to a reduction in N loss (Edwards *et al*. 2007). Feeding high carbohydrate and low protein crops, such as fodder beet (FB), has therefore been suggested as a potential method to reduce urine-N concentration and subsequently reducing N losses (Dalley *et al*. 2017). The relationship between WSC:CP and urine N concentration is shown in Figure 8.

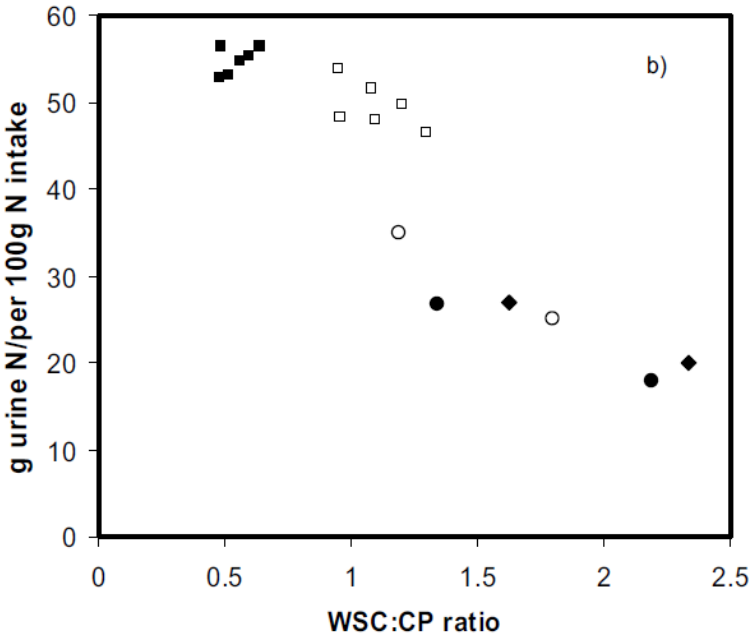

Figure 8. Combined data from a range of both UK (IGER) and Dutch studies showing a continuum between N utilisation efficiency for urine in dairy cows, in relation to the WSC:CP ratio of the forage component of the diet offered. Data sources: ■ 2001 data from Tas *et al*. (2005); Tas *et al*. (2006); □ 2000 data from Tas *et al*. (2005); Tas *et al*. (2006); ○ Miller et al. (2001a); • Miller et al. (2001b); ◆ Moorby *et al*. (2006). Figure adapted from Edwards *et al*. (2007).

Totty *et al*. (2013) also found that cattle on a diet of diverse pasture (chicory (*Cichorium intybus*), plantain, lotus (*Lotus pedunculatus*), high sugar RG and WC) had 17-19% lower urinary-N content compared with PRG and WC diets. This diverse diet had higher levels of WSC and lower CP, likely leading to better rumen N use efficiency. This shows the potential for manipulating WSC and CP levels in plants to reduce N losses.

Compounds such as condensed tannins (CT) can be found in plants. These compounds can alter urine and faecal N compositions. Condensed tannins form complexes with proteins in the rumen, protecting them from microbial digestion. This results in either more efficient digestion of amino acids in the lower intestine and abomasum, or the excretion of the CT protein complex in the dung (Min *et al*. 2003; De Klein & Eckard 2008). Faecal-N is much less susceptible to short term N loss, with the N_2O emission factor for faecal-N being 25% of that of urine (Luo & Kelliher 2010). An increased faecal: urine N ratio is seen as desirable from an environmental perspective. Grainger *et al*. (2009) reported that when CTs were added to the diet of lactating cows it resulted in a 45- 59% reduction in urinary N excretion and an 18-21% increase in N in the faeces. Currently, CT extracts are expensive and are not an efficient way of reducing N losses, however, many forage plants contain CT. Plant breeding may be an effective way to introduce CT into the diets of animals, however, further research is required to identify suitable and cost effective high CT forages (Eckard *et al*. 2010).

It has been suggested that increasing the proportion of non-urea N compounds (NUNC) in animal urine could reduce N losses (Gardiner *et al*. 2016). These NUNCs include allantoin, hippuric acid (HA), creatine, creatinine, ammonia, amino acids, uric acid, xanthine and hypoxanthine (Topps & Elliott 1967). This reduction in N losses could occur if soil organisms could not transform these NUNCs to NH_4^+. However, there are well recognised pathways for NUNC catabolism in soils, making it unlikely to be an effective way to reduce N losses (Gardiner *et al*. 2016). HA has been shown in a laboratory environment to be a potential inhibitor of N_2O emissions. As a derivative of HA, benzoic acid is a known microbial agent; however, this reduction in N_2O losses has not been shown in *in situ* field studies (Kool *et al*. 2006; Bertram *et al*. 2009; Clough *et al*. 2009; Krol *et al*. 2015). The likelihood of NUNCs prevailing as inhibitors is low due to their potentially rapid degradation in soil to benign compounds (Gardiner *et al*. 2016). More research is needed to investigate the potential of manipulating cattle diets, to alter urine-N loading rate, as a tool to reduce N losses, in shallow stony soils.

Urine-Carbon

Research has also shown that altering cattle and sheep diets can affect urine-C losses (Chandramoni *et al*. 1999; Sahoo *et al*. 2002; Hales *et al*. 2012, 2013). Hales *et al*. (2013) also showed that by adding wet distillers grains soluble (WDGS) to cattle diet cattle, consisting of steam flaked corn (SFC), urine C losses could vary from 21 g C day^{-1} cattle $beast^{-1}$ (100% SFC. 0% WDGS) up to 38 g C day^{-1} cattle $beast^{-1}$ (55% SFC. 45% WDGS). Hales *et al*. (2012) also showed the addition of 30% WDGS to di*et al*tered cattle urine-C losses from 18.6 g C day^{-1} cattle $beast^{-1}$ up to 25.2 g C day^{-1} cattle $beast^{-1}$. However, there is currently no published research that describes the effects of contrasting cattle diets on cattle urine-C concentrations and subsequent N leaching losses.

An important form of C that could potentially be found in urine is BNIs. Currently, there is limited literature on BNIs in urine (Luo *et al*. 2015b; Di & Cameron 2016; Judson *et al*. 2018; Yao *et al*. 2018). Di *et al*. (2016) and Yao *et al*. (2018) both suggested that FB urine contains compounds which affect the N transformation processes, after finding a reduction in N_2O emissions under FB urine treatment, compared to that for a kale urine treatment. The N_2O emissions from cow urine from FB were 39% lower than urine from kale with the same urine-N application rate (300 kg N ha^{-1}) (Di *et al*. 2016). Luo *et al*. (2015b) found a reduction in the N_2O emission factor in urine from sheep grazing forage rape (*Brassica napus L*.) compared to that for a PRG urine treatment and suggested that the forage rape urine contained plant secondary metabolites that affected N transformation processes. A BNI in urine was also suggested by Judson *et al*. (2018); who found that urine from sheep fed plantain diets reduced the rate of nitrification in soil compared with urine from sheep fed PRG, throughout the 28 days after application. These reductions in N losses are highly significant due to the potential economic and environmental benefits. However, the reason behind these reductions in N losses and the interactions

between the C and N cycle are largely unclear. More work is needed to elucidate the mechanisms behind these reductions in N losses under FB urine.

More research is needed to investigate the potential of manipulating cattle diets, to alter urine C and N concentrations and compositions, as a tool to reduce N losses.

2.5.6 Bio-char

Bio-char is a C rich product derived from the pyrolysis of organic material at low temperatures (<700^{o}C) (Clough & Condron 2010; Clough *et al*. 2013). This process mirrors the formation of charcoal. The organic materials that undergo pyrolysis to produce bio-char can be organic waste products, such as forestry waste, agricultural residues, farm manures and urban organic wastes. Bio-char properties can be considerably influenced by the type of feedstock and pyrolysis conditions (Arbestain *et al*. 2016). Bio-char has been suggested to enhance NH_3 and NH_4^+ retention and reduce N_2O and NO_3^- losses, however, literature suggests these results are variable (Clough & Condron 2010; Chen *et al*. 2013; Clough *et al*. 2013). Although the literature review indicated that further research into the effect of bio-char on the N is needed; this project will not focus on bio-char.

2.6 Synthesis of literature review findings and objectives

A review of the literature has identified the following key knowledge gaps:

- There is very little information on the effects of organic amendments on N losses and transformations in urine patches, in shallow stony soils.
- Research is needed to investigate and quantify the effect of winter plant growth on the N cycle, in shallow stony soil.
- More research is needed to investigate the potential of manipulating cattle diets, to alter urine-C and urine-N loading rate, as a tool to reduce N losses, in shallow stony soils.
- Little research has been done on New Zealand winter forage, lucerne and pasture systems in relation to plant exudates and their impact on the N cycle. Further work on plant BNI effects on N losses is urgently needed to reduce environmental impacts of pastoral farming.
- There has been no research on the effect of Italian and plantain on reducing N losses from shallow stony soils.

Therefore this research program had the following objectives:

Objective 1: To determine the effect of adding readily available C on N losses (NO_3^- leaching and N_2O emissions) from urine treated shallow stony soil and the effect of the C on the key soil N processes of immobilisation and nitrification (including AOB abundance) in urine patches.

Objective 2: To determine the effect of winter plant growth on N losses from urine-treated shallow stony soil and the effect on the key soil N processes.

Objective 3: To determine the effect of manipulating cattle diet on N losses from urine-treated shallow stony soil and the effect on the key soil N processes.

Objective 4: To determine the effect of winter forage, lucerne and pasture systems on N losses and the effect on key soil N processes (in particular AOB abundance), in shallow stony soils.

Objective 5: To quantify the effect of plantain in pasture and the effect of urine from cows grazing on plantain, on N losses and the effect on key soil N processes, in shallow stony soils.

Objective 6: To quantify the effects of plantain and IRG on N leaching losses from urine-treated shallow stony soil, and the effect on the key soil N processes, over a range of urine application dates.

Chapter 3

General materials and methods

3.1 Site description

The lysimeter/soil block experiments were conducted at the Ashley Dene Research and Development Station (ADRDS), near Lincoln, Canterbury (43°38'51.2"S 172°20'45.5"E; 17 m above sea level). This site has an average annual rainfall of 618 mm, an average temperature of 11.3°C (Macara 2016), and very stony, shallow, free draining soils (Appendix D, E). These conditions are typical for a majority of the Canterbury region in New Zealand. The trial was performed in a lysimeter trench facility at ADRDS (Figure 10).

3.2 Lysimeter/soil block collection and installation

3.2.1 Collection sites

For the trials, undisturbed soil monolith lysimeters, 500 mm in diameter and 700 mm deep, were used. Undisturbed soil monoliths were used to ensure soil water dynamics were not disturbed (Carrick *et al*. 2017). The soil in these lysimeters consisted of Balmoral stony silt loam (Pallic Firm Brown soil) (Landcare Research 2016a), Udic Haplustept (Soil Survey Staff. 2014). This soil is very stony, shallow, free draining and typical of soils used for dairying in the Canterbury region of New Zealand (Carrick *et al*. 2013). The key physical properties of the soil profile are provided in Table 4. The collection sites of the lysimeters are described in Appendix E.

Table 4. Key physical properties of the Balmoral stony silt loam. Adapted from Carrick *et al.* (2017)

			Fine fraction < 2 mm			% Stones (w/w)
Horizon	Horizon depth (cm)	Texture class	% Sand	% Silt	% Clay	Whole soil fraction
Ap	28	Silt loam	29	53	18	55
Bw	70	Sand	86	6	7	71
C	140+	Sand	94	3	3	75

3.2.2 Lysimeter/soil block collection and installation

The lysimeters were collected, following well-established protocols and procedures (Cameron *et al*. 1992). This involves placing a metal cylinder casing on the soil surface. The soil around the casing is then dug away in 20 cm depth increments. Care is taken to reduce disturbance to the soil structure

within the casing. The casing is gradually pushed down in small increments as the surrounding soil is removed. Once the casing has reached the desired depth, the gap between the soil column and casing is sealed with melted Vaseline. The lysimeter is then cut from the subsoil at the bottom, using a cutting plate, which is then secured to the lysimeter casing. The lysimeters are then transported on a specially designed trailer with air bag suspension, thus minimising the risk of disturbance to the soil. The pasture and lucerne lysimeters had a small layer of gravel added to the bottom of the soil column, to prevent blockages at the bottom. It was not possible to add a layer of gravel to the FB lysimeters, due to the protruding FB bulbs. However, the free draining, gravelly nature of the soil at the bottom of the profile, meant there was very low risk of blockage.

Between collection and installation, the lysimeters were maintained to ensure they experienced typical paddock conditions. This involved harvesting the pasture and lucerne using shears to simulate grazing, applying N fertiliser to all lysimeters and watering of all the lysimeters to simulate irrigation, ensuring the plants did not suffer water stress.

A corresponding soil block (500 mm in diameter and 200 mm deep) was installed next to each lysimeter. The soil blocks were collected from the same location using a metal ring. The metal ring was pushed down into the soil profile, while the soil around the ring was removed, to minimise disturbance of the soil profile within the ring. Once the ring reached the required depth (200 mm), the soil block was cut at the base using a cutting plate. The block was then transported and installed next to its corresponding lysimeter at the trench facility. Typical collection and installation of a soil block is shown in Figure 9.

Figure 9. a) A soil block dug to the required depth of 200 mm, b) a soil block being transported, and c) a soil block placed next to its corresponding lysimeter.

Once the lysimeters were installed, the surrounding area was back filled with sand at the lower depths and top soil around the top layer. The soil blocks were positioned on a bed of sand and the

lysimeters/soil blocks were surrounded with top soil. The top soil was levelled to the same height as the soil in the lysimeters/soil blocks. The installation process is shown in Figure 10.

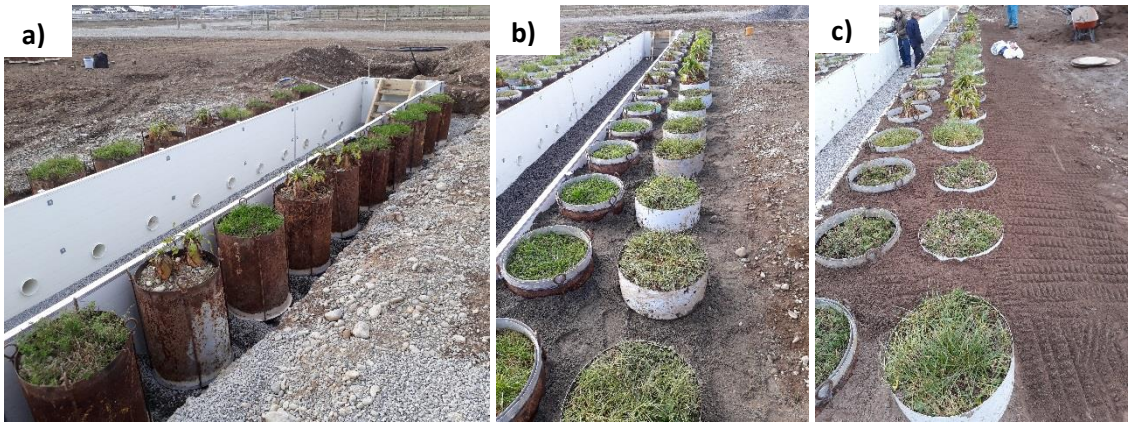

Figure 10. a) After installation of the lysimeters in the trench facility, b) after back filling with sand and installation of soil blocks, and c) after application of top soil to surround the lysimeters.

3.2.3 Treatment application

Simulated winter grazing

To ensure the treatments were applied to lysimeter/soil blocks, under typical winter farm conditions, simulated winter grazing was performed. This involved the harvesting of plants and simulating cattle trampling, prior to treatment application, which are described below.

Plant harvest

The pasture lysimeters/soil blocks were trimmed to a residual mass equivalent of 1500 kg DM ha^{-1} using electric hand shears, to simulate winter grazing (Figure 11a). The cut pasture was removed from the pasture lysimeters/soil blocks, as it would be during grazing. The FB plants were removed from the lysimeters/soil blocks to simulate winter grazing by stock (Figure 11b). The lucerne lysimeters/soil blocks did not undergo harvesting as they had very little late autumn/winter growth.

Figure 11. a) Using electric hand shears to simulate winter grazing on a pasture soil block, down to 1500 kg DM ha, and b) the removal of a fodder beet plant from a soil block.

Simulated trampling

Simulated trampling of the lysimeters was performed (Figure 12). The soil in all lysimeters was trampled using cow hoof simulation equipment designed to provide approximately 200 kPa pressure; similar to the pressure exerted by an adult cow hoof (Di *et al*. 2001). As the FB is grazed at a higher stocking intensity, the FB lysimeters/soil blocks each received 45 'stomps'. Lucerne and pasture are grazed at a lower stocking rate, so the lucerne and pasture received only 6 'stomps' each.

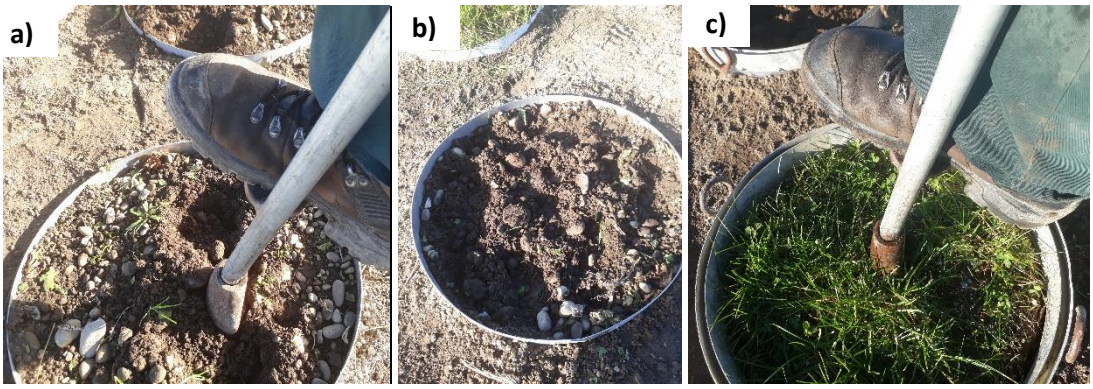

Figure 12. a) The simulated cow hoof during grazing on a fodder beet soil block, b) the final result of the simulated grazing on a fodder beet soil block, and c) the simulated hoof during grazing on a pasture lysimeter.

Urine

Each lysimeter/soil block had 2 L of cattle urine applied using a rose head watering can, allowing an even spread of urine over the lysimeter/soil block (Figure 13).

Figure 13. Application of urine to the lysimeters.

3.2.4 Collection, sampling and analysis

Leachate collection and analysis

Leachate was collected from each lysimeter after every drainage event, using plastic tubing attached to an outlet nozzle at the bottom of the lysimeters, and leading to 10 L collection containers. Leachate volumes were measured and 50 mL samples were taken for chemical analysis. Leachate samples were

taken “mid-stream” (Figure 14). Samples were kept frozen at -20 °C until they were analysed for NH_4^+ and NO_3^- using a FOSS FIAstar 5000 twin channel analyser with SoFIA software version 2.00 (Gal *et al*. 2004). Leachate samples were also analysed for C content (Organic and Inorganic) using a Shimadzu Total Organic C analyser (TOC-5000A) fitted with a Shimadzu ASI-5000A autosampler.

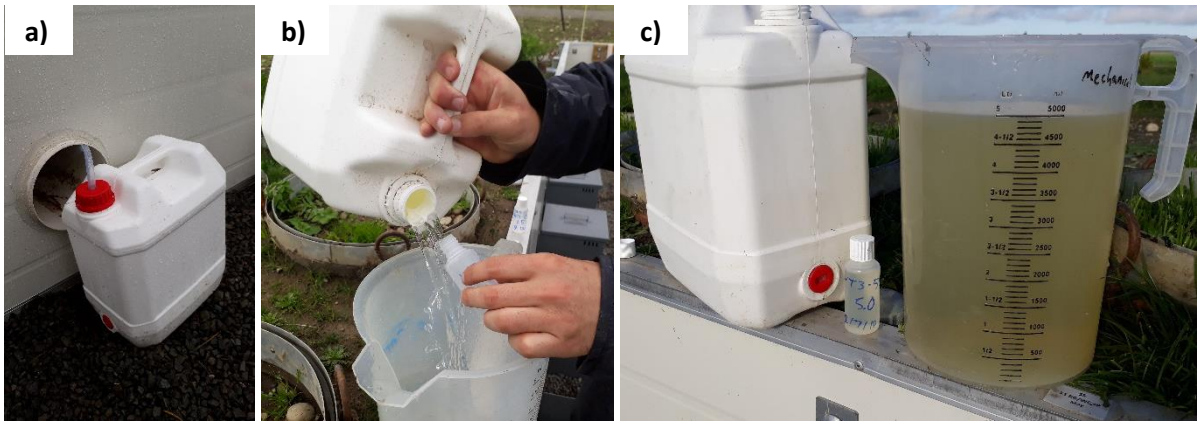

Figure 14. a) A collection container and plastic tubing attached to the bottom of the outlet nozzle, b) a leachate sample being collected “mid-stream”, and c) a collection container, 50 mL leachate sample and measuring jug.

Greenhouse gas emission sampling and analysis

For the collection of the greenhouse gas (N_2O, CH_4^+, CO_2) samples, a closed chamber method similar to that described in Hutchinson and Mosier (1981), was used (Figure 15). The gas chambers used were constructed from a metal cylinder insulated with 25 mm thick polystyrene foam to avoid heating of the atmosphere in the chamber during sampling. During the sampling process the chamber was placed inside a small water trough which was mounted around the top of each lysimeter casing. The chamber remained there for 40 minutes, during which time three 20 mL samples were collected, 20 minutes apart, using a syringe through a rubber septum on top of the gas chamber. The N_2O, CH_4^+ and CO_2 concentrations of the samples were analysed using a gas chromatograph (Model 8610C, SRI Instruments, California, USA) linked to a Gilson GX-271 autosampler (Gilson Inc, MI, USA).

Additional samples were collected for ^{15}N tracer analysis, once a week, following the regular gas sampling (as described above). The chambers remained on the lysimeters for a total of 3 hours, after which a larger (40 mL) sample was collected. These samples were analysed for ^{15}N tracer concentrations in the N_2O gas, using isotope ratio mass spectrometry (IRMS) (Sercon Ltd, Crewe, CW1 6JT, UK).

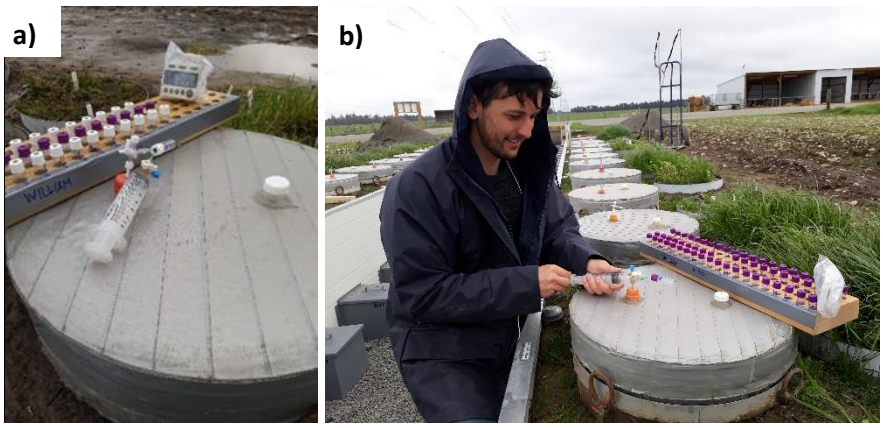

Figure 15. a) A gas chamber and sample collection equipment on top of the lysimeter, and b) a gas sample being collected.

Herbage collection and analysis

Once pasture plant development had reached the 2-3 leaf stage and yields were on average 3000 kg DM ha^{-1} the pasture lysimeters were harvested. Herbage was cut to a residual height of approximately 50 mm (1500 kg DM ha^{-1}) (Figure 16). The herbage from all the soil blocks was discarded.

The lucerne lysimeters were harvested according to the following seasonal management guidelines (Moot *et al*. 2003), which resulted in six harvests:

Spring (August-November): Graze when herbage 200-250 mm high (1500 kg DM ha^{-1}), allow 5-6 weeks regrowth (350-450 mm height, ~3000 kg DM ha^{-1}).

Summer (December-January): 30-35 day rotation.

Autumn (February-March): Long rotation. Allow flowers on at least 50% of the lucerne stems sometime between mid-summer to autumn to encourage root recharge.

Winter (June-July): Final hard graze early winter (first week of June). Leave lucerne until spring to develop new shoots.

The pasture/lucerne herbage harvested were collected into separate paper bags. They were then weighed fresh, oven-dried at 70°C for 72 hours and then reweighed to calculate the dry matter production.

The FB plants were harvested by removing them from the ground by hand and washed to remove soil. The leaves were separated from the bulb. The leaves were collected into separate paper bags, weighed fresh, oven-dried at 70°C for 72 hours and then reweighed to calculate the dry matter production. The bulbs were weighed fresh, sliced (Figure 16), oven dried at 70°C for 6 days and then reweighed to calculate the dry matter production.

Figure 16. a) Pasture being harvested using electric hand shears, b) fodder beet being removed by hand, and c) fodder beet sliced and ready for drying.

The dry pasture, lucerne and fodder beet samples were then ground using a Retsch Ultra Centrifugal Mill ZM 200, with a 1 mm sieve and running at a speed of 18,000 rpm. Care was taken to thoroughly clean the grinder in-between each sample. The samples were stored in sealed 70 mL containers at room temperature in the dark, until analysis was performed.

The samples were analysed for their total C & N contents using an Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany). During analysis the samples were combusted at 900°C in an a oxygen atmosphere. The combustion process converts any elemental C and N into CO_2, N_2 and NO_x. The NO_x is subsequently reduced to N_2. The gases were then passed through a TC (thermal conductivity) cell to determine CO_2 and N_2 quantities. This allowed the total C content and N uptake of the pasture/lucerne to be calculated.

Soil sampling and analysis

Destructive soil sampling was conducted from the soil blocks to provide insight into the N processes occurring in the soil. The blocks had soil samples taken on days 0, 1, 7, 14, 28, 56 and 112 after urine application. The samples were taken using a soil corer (100 mm depth, 75 mm diameter). The resulting holes were filled in with sand and marked to ensure they were not resampled (Figure 17). The soil samples were stored at -80 °C until analysis. The samples were analysed for NH_4^+, NO_3^- concentrations, using a FOSS FIAstar 5000 twin channel analyser (Foss Tecator AB, Hoganas, Sweden).

Figure 17. a) Soil sample collection, b) the hole after soil sample collection being filled with sand, and c) the sand filled areas with white location markers in them.

DNA analysis of soil samples

DNA extraction

The DNA extraction and polymerase chain reaction (PCR) analysis followed methodologies adapted from Di *et al*. (2009b). DNA was extracted from the soil using a NucleoSpin® Soil Kit (Macherey-Nagel, Düren, Germany) as per the manufacturer's instructions. In brief, a 0.25 g soil sample was weighed into a NucleoSpin® Bead Tube to which 700 µL of Buffer SL2, and 150 µL of Enhancer SX were added. The sample was homogenised for 1 min using a FastPrep®-24 Sample Preparation System (M.P. Biomedicals, California, USA) at a speed of 6 m s^{-1}. The tubes were centrifuged at 11,000 rpm for 2 min (Centrifuge 5424, Eppendorf AG,Hamburg, Germany), and the supernatant transferred into a sterilised 1.7 mL tube. Next 150 µL of Buffer SL3 was added and the samples were shaken for 5 s prior to incubation at 4°C for 5 min. The tubes were centrifuged at 11,000 rpm for 1 min, and up to 700 µL of supernatant was transferred into a NucleoSpin® Inhibitor Removal Column fitted on top of a collection tube. Tubes were again centrifuged at 11,000 rpm for 1 min and the column was discarded. 250 µL of Buffer SB was added to the flow through and mixed with a pipette. A NucleoSpin® Soil Column was placed in a new collection tube and a 550 µL sample of this was loaded onto the column. This was centrifuged at 11,000 rpm for 1 min and the flow through discarded. This step was repeated until no sample remained. 500 µL of Buffer SB was added to the NucleoSpin® Soil Column. This was centrifuged at 11,000 rpm for 30 s and the flow through was again discarded. The column was then washed with 550 µL of Buffer SW1, and twice with 700 µL of Buffer SW2. Once the final flow through was discarded, the column and collection tube was centrifuged at 11,000 rpm for 2 min to remove any residual ethanol. The NucleoSpin® Soil Column was then transferred to a new collection tube and the DNA was eluted using 100 µL of Elution Buffer SE. The sample was incubated at room temperature for 1 min and centrifuged at 11,000 rpm for 30 s. DNA was stored at −20°C for further analysis.

q-PCR

The AOB *amoA* genes were quantified using the primer pairs Arch-amoAF and Arch-amoAR (Francis *et al.* 2005), (Table 5). A reaction mixture of 16 µL contained 8 µL 2x SYBR® Premix Ex Taq™ (Tli RNaseH Plus, Takara Bio Inc., Shiga, Japan), 0.4 µL of each primer, and sterile deionised water to bring up to total volume of 14.5 µL and 1.5 µL of DNA sample. All genomic DNA samples were diluted 10 times with deionised water prior to use. Serial dilutions of standards with a range of 101–107 copies μL^{-1} were run in duplicate for each gene to produce standard curves. Once the PCR reactions were prepared the RotorDisc™ 100 was sealed using a Gene-Disc™ Heat Sealer (HS-01, Corbett Research, Australia). The q-PCR temperature profiles used are given in Table 5. A melting curve analysis was performed after amplification to check for nonspecific amplification products. The fluorescence was measured continuously as the temperature increased from 72°C to 99°C. Data were then analysed using the Rotor-Gene™ series software 1.7.

Table 5. q-PCR primers and temperature cycles.

	Gene	AOB *amoA*	
	Primer pairs	amoA-1F 5'-GGGGHTTYTACTGGTGGT-3' (Stephen *et al*. 1999) amoA R-i 5'-CCCCTCNGNAAANCCTTCTTC- 3' (Hornek *et al*. 2006)	
# of cycles	Cycling conditions	Temperature (°C)	Time (s)
1	Initial denaturation	94	120
40	Denaturation	94	20
	Primer annealing	57	30
	Extension	72	30
	Final extension	72	180

Standard curves for real-time qPCR were developed using the following process. Bacterial and archaeal *amoA* genes were amplified from the extracted DNA using the aforementioned primers. A qPCR clean up kit (Axygen) was used to purify the PCR products which were cloned into the pGEM-T Easy Vector (Promega, Madison, WI). Following the manufacturer's instructions, the resulting clones were transformed in *Escherichia coli* JM109 competent cells (Promega). The transformed *E. coli* cells were grown on solid LB plates at 37°C overnight. Ten to 15 bacterial colonies from the plate were individually inoculated into a 3 mL LB broth medium and incubated overnight in an orbital incubatorshaker at 37°C and 250 rpm. The plasmids carrying correct gene inserts were then extracted from bacterial cultures using QIA Prep Spin Miniprep Kit (Qiagen, Crawley, UK) and sent for sequencing. The DNA concentration was determined on a Qubit™ Fluorometer (InvitrogenTM, New Zealand). The copy

numbers of target genes were calculated directly from the concentration of extracted DNA. To generate an external standard curve, tenfold serial dilutions of a known copy number of the extracted plasmid DNA were subjected to a real-time PCR assay in duplicate.

^{15}N tracer analysis

^{15}N enrichment was measured in leachate, gas and herbage samples (sample collection previously described) using a continuous flow IRMS.

For analysis, the N present in the leachate samples was concentrated on 7 mm glass fibre disks using the diffusion procedure described by Brooks *et al*. (1989). In brief this involved the reduction of NO_3^- to NH_4^+ and then the conversion of all the NH_4^+ to ammonia (NH_3). This NH_3 diffused into the headspace of the sealed sample container where it was collected on acidified 7 mm glass fibre filter paper disks (MicroScience MS GD, 47 mm) suspended on a wire above the liquid sample in the sealed container, over 6 days (Figure 18). The filter papers were first cleaned by washing in 2M KCl solution three times, followed by deionised water three times then dried and punched into 7 mm disks. These disks were acidified with 10 µL of 2.5 M $KHSO_4$ no more than 5 minutes before capping the container.

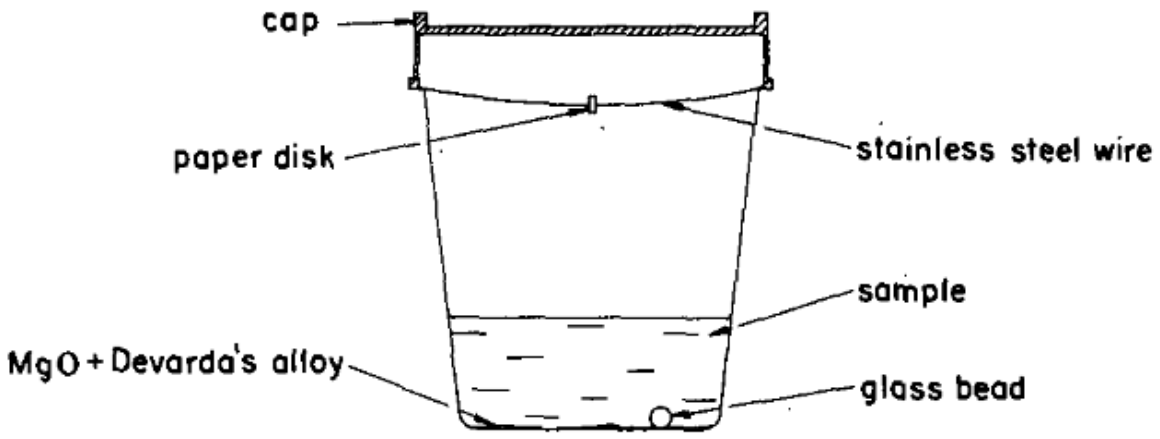

Figure 18. Diffusion apparatus used to prepare soil extracts for ^{15}N analyses. From Brooks *et al*. (1989).

The volume of the leachate sample diffused varied, depending on the NO_3^--N and NH_4^+-N concentrations (previously determined by FIA), so that there was 100 µg of N on the disk. Deionised water was added to make the total volume 50 mL. Next, 0.1 mL of 21% w/v Brij-35 solution, 0.4 g of Devarda's alloy, and 0.2 g of MgO were added. The container was immediately capped and gently mixed with the help of two acid-washed glass beads in the container, care was taken not to splash the solution on the disk. After 6 days the disks were dried in a desiccator for at least 24 hours, and then sealed in tin capsules ready for IRMS analysis. Deionised water blanks and two standard were included in each run and were replicated three times.

The previously ground herbage samples were also weighed into tin capsules. These solid samples (herbage and diffused leachate), were initially combusted at 1000 °C in an oxygen atmosphere in an automated Dumas-style elemental analyser which was linked to the 20-22 stable IRMS (Sercon Ltd, Crewe, CW1 6JT, UK).

Gas samples were analysed using a TGII trace gas system, equipped with cryo-trapping and focusing, to isolate the fraction of interest. The ^{15}N enrichment of the samples were analysed on the 20-22 stable IRMS (Sercon Ltd, Crewe, CW1 6JT, UK).

^{15}N balance calculations

Percentage ^{15}N recoveries in soil, herbage, leachate and N_2O emissions were calculated using the equation from Cabrera and Kissel (1989):

$$\%^{15}N\ recovery = 100 \times \frac{p(c-b)}{f(a-b)}$$

where:

$\%^{15}N$ recovery	$=^{15}N$ in measured fraction as a percentage of the ^{15}N applied
p	= moles of N
c	= atom% ^{15}N enrichment of the sample (from IRMS)
b	= atom% ^{15}N in Control (non-urine treated) fraction
f	= moles of N in the urine applied to the lysimeter
a	= atom% ^{15}N enrichment (10%)

A value of 0.3663% was used as the b value (atom% ^{15}N natural abundance enrichment).

Leachate

The N leaching loss (g) for each lysimeter was calculated by multiplying the mineral N (NO_3^- + NH_4^+) concentration (mg N L^{-1}) by the drainage volume on each sampling occasion, and dividing by 1000. This value was used to determine the total moles of N leached from each lysimeter by dividing the total mass of N leached from each lysimeter by the molar mass of N (14.0067 g mol^{-1}). Interpolation was used to estimate the atom% ^{15}N enrichment for those sampling occasions where ^{15}N analyses were not performed. Nitrogen ^{15}N recovery was determined using the equation above for each leaching event, the values were summed to give the total ^{15}N recovery in leachate during the experimental period.

Herbage

For each lysimeter, the mass of N (g) in herbage at each harvest was determined by multiplying the dry matter harvested (g) by the N content (%) in the herbage, measured by the Elementar Vario-Max

CN Elemental Analyser (Elementar GmbH, Hanau, Germany) and dividing by 100. The moles of N at each harvest were then determined by dividing the mass of N by the molar mass of N (14.0067 g mol^{-1}). Herbage samples from a select number of harvests were analysed by IRMS, interpolation was used to estimate the ^{15}N enrichment for those sampling occasions where ^{15}N analyses were not performed. Nitrogen ^{15}N recovery was determined using the equation above for each harvest, then these were summed to determine the total ^{15}N recovery in herbage during the experimental period.

Nitrous oxide emissions

The moles of N evolved as N_2O (moles N_2O-N $lysimeter^{-1}$ day^{-1}) were determined from the N_2O flux (mg N m^{-2} h^{-1}), by first converting to units of g N $lysimeter^{-1}$ day^{-1} and then dividing by the molar mass of N (14.0067 g mol^{-1}). Nitrogen ^{15}N enrichment was determined for three N_2O sampling dates, so interpolation was used to estimate the N_2O-^{15}N enrichment for those sampling occasions where ^{15}N analyses were not performed. Nitrogen ^{15}N recovery was determined for each sampling date, then integration was used to determine the total recovery of N_2O-^{15}N emitted during the measurement period.

Chapter 4

Lysimeter experiment 1

4.1 Introduction

Nitrogen is very important to New Zealand agriculture, with N a key nutrient needed for plant growth. However, N can be lost to the wider environment through NO_3^- leaching and N_2O emissions. These losses are detrimental to the environment, due to the impact NO_3^- has on freshwater quality and, the greenhouse gas and ozone depleting properties of N_2O. There is also a significant economic loss, as the lost N needs to be replaced by farmers through fertiliser application. It is therefore necessary to improve understanding of the factors that affect these losses, and develop potential strategies to reduce these losses. One of these important factors are the interactions between the C and the N cycle. These interactions include immobilisation/mineralisation, nitrification, and denitrification, all of which are influenced by C inputs. Currently, there is a lack of understanding of these important interactions, and further research is required.

Therefore, Lysimeter experiment 1 aimed to elucidate the interactions between C and the N cycle. This was done by manipulating C inputs (using different crop types, urine addition from animals fed on different crop types, and artificial C inputs) and then measuring the effects these manipulations had on the N cycle. This experiment was split into two trials; Trial 1 (Talbot *et al*. 2019) focused on the effect artificial inputs of readily available C has on the N cycle; Trial 2 (Talbot *et al*. 2020) looked at the effect different urine compositions and crop types have on the N cycle.

4.2 Trial 1 (Talbot *et al*. 2019)

4.2.1 Research article

This section has been published in the New Zealand Journal of Agricultural Research, published by Taylor & Francis.

Effects of adding readily available carbon to soil on nitrogen losses from cattle urine patches

Abstract

A lysimeter experiment was carried out to investigate the effects of applying readily available carbon (12 or 24 t sucrose ha^{-1}) to soil on nitrogen (N) losses from cattle urine patches. The carbon (C) was readily available to microbes and was applied onto intact soil monolith lysimeters, containing stony silt loam soil, beneath either perennial ryegrass (*Lolium perenne*)/white clover (*Trifolium repens*) pasture (PRG/WC) or lucerne (*Medicago sativa*). Cow urine (700 kg N ha^{-1}) was applied in early July 2017, two days after C application. The application of readily available C increased the immobilisation of N in the soil which reduced the amount of soil mineral N. The C-induced immobilisation of N reduced the ammonia oxidising bacteria population growth rate and the amount of nitrate leached by 51-89%. The addition of readily available C had no significant effect on nitrous oxide emissions. These findings were consistent under both PRG/WC and lucerne.

Introduction

In traditional New Zealand dairy farming systems, cattle graze outside year-round, depositing urine onto the soil. These urine patches have high loading rates of nitrogen (N) (700 – 1000 kg N ha^{-1}) (Haynes & Williams 1993; Selbie *et al*. 2015). Nitrogen is a key nutrient needed for plant growth. However, the high rate of N deposited in urine often exceeds plant requirements. The excess N can be lost to the wider environment through nitrate (NO_3^-) leaching and nitrous oxide (N_2O) emissions (Selbie *et al*. 2015). The lost N can have serious negative environmental, health and economic impacts. The leached NO_3^- can enter into waterways and ground water and causes eutrophication (Smith *et al*. 1999). Eutrophication leads to increased growth of algae and other aquatic plants, which can be unsightly and create problems for freshwater recreational activities. There is also a health risk from increased NO_3^- concentrations (i.e. >11.3 ppm NO_3^--N) in drinking water (Grizzetti *et al*. 2011). The ingested NO_3^- is converted into NO_2^- in the stomach and this can be absorbed into the bloodstream reducing the blood's oxygen carrying capacity, which can potentially cause death from cellular anoxia. The lost N must also be replaced by farmers through fertilisers adding an extra economic cost. Farm N_2O emissions are also of great significance, as N_2O is a potent greenhouse gas. The long term

global warming potential of N_2O is 265 times that of carbon dioxide (CO_2) (Myhre *et al*. 2013; Pachauri *et al*. 2014). It has also been shown that N_2O has become, and will remain, the biggest threat to the ozone layer in the 21st century (Ravishankara *et al*. 2009). It is therefore necessary to improve understanding of the factors that affect these N losses and develop potential strategies to reduce them.

In the past two decades there has been increasing intensification of agriculture in Canterbury, with large numbers of sheep farms converted to dairy farms (Dynes *et al*. 2010). However, in Canterbury the soil is predominately free draining, shallow and stony and therefore highly susceptible to N leaching (Carrick *et al*. 2013). A potential strategy for reducing N losses from agriculture is through the addition of carbon (C). Carbon interacts with the N cycle through immobilisation/mineralisation, nitrification, and denitrification. These interactions have the potential to be manipulated in ways that could reduce N losses from agricultural systems. Carbon can be added to the soil through plant root exudates. Plants can release nearly 25% of the C fixed photosynthetically into the rhizosphere (Marschner 1995; Walker *et al*. 2003; Stockmann *et al*. 2013). Carbon can also be added to the soil through artificial inputs. The application of artificial C inputs (e.g. sawdust, sucrose, glucose) have been used in some studies to increase the rate of N immobilisation and subsequently to reduce N leaching (Kanal 1995; Sarrantonio 2003; Chaves *et al*. 2005; Szili-Kovács *et al*. 2007; Chaves *et al*. 2008; Shepherd *et al*. 2010). Few studies have examined the combined effects of C addition on key soil processes (e.g. immobilisation, nitrification), soil microbial communities and N losses (e.g. NO_3^- leaching, N_2O emissions) under animal urine patches.

This study therefore will determine the effect of adding C that is readily available to microbes on N losses (NO_3^- leaching and N_2O emissions) from urine treated soil and the effect of the C on the key soil processes of immobilisation and nitrification (including ammonia oxidising bacteria abundance) in urine patches. Sucrose was used as the source of C because it was readily available to microbes and allowed the trial results to be compared to other data reported in the literature (e.g. Shepherd *et al*. (2010)). This study was carried out using monolith lysimeters and soil blocks, under two different crops: perennial ryegrass/white clover (PRG/WC) and lucerne. These two common crops have varying plant characteristics including different root depths, root exudates and winter growth rates (Baars *et al*. 1975; Evans 1978; McKenzie *et al*. 1990; Neumann & Römheld 2007).

Materials and methods

Lysimeter and soil block collection and installation

The experiment was conducted at the Lincoln University Ashley Dene Research and Development Station (ADRDS), near Lincoln, Canterbury (43°38'51.2"S 172°20'45.5"E; 17 m above sea level). For this experiment, 25 undisturbed soil monolith lysimeters, 500 mm in diameter and 700 mm deep, were

used. Fifteen were collected from a perennial ryegrass (*Lolium perenne*) cv. 'Prospect'/white clover (*Trifolium repens*) cv. 'Legacy' pasture (PRG/WC) paddock (43°38'37.7"S 172°20'38.8"E), while the remaining 10 were collected from a lucerne (*Medicago sativa*) cv. 'Stamina 5' paddock (43°38'51.8"S 172°21'01.0"E). The lysimeters were collected in the summer of 2016/2017 following well-established protocols and procedures (Cameron *et al*. 1992). Between their collection and installation, the plants on the lysimeters were maintained to ensure they replicated typical paddock conditions. This was done through harvesting the pasture/lucerne with electric shears to simulate grazing, and watering of the lysimeters to simulate farm irrigation practices. Each lysimeter also had a corresponding soil block, 500 mm in diameter and 200 mm deep to allow periodic removal of soil samples. These soil blocks were collected in June 2017 from the same location as their corresponding lysimeters. The lysimeters/soil blocks were installed into a lysimeter trench facility at the ADRDS in June 2017. Once the lysimeters were installed, the area around them was back filled with sand at the lower depths and top soil around the top layer. The soil blocks sat on a bed of sand and were surrounded with top soil. The top soil was levelled to the same height as the soil in the lysimeters/soil blocks.

The soil in the lysimeters/soil blocks was a Lismore/Balmoral stony silt loam (Pallic Firm Brown soil) (Landcare Research 2016b, a), Udic Haplustept (Soil Survey Staff. 2014) (Table 4). This soil is very stony, shallow, free draining and typical of soils used for dairying in the Canterbury region of New Zealand. Soil samples were taken at depths of 0-100, 100-200, 200-300, 300-450 and 450-700 mm at both collection sites. The samples were placed in clear plastic bags, sealed, labelled and sent for a basic soil analysis at Analytical Research Laboratories (Ravensdown, New Zealand) (Table 6).

Fertiliser was applied to the PRG/WC lysimeters and soil blocks on the 30th June 2017. Fertiliser was applied because the Olsen P value (15) and the pH (6) of the PRG/WC soil (0-75 mm depth) test were lower than typical dairy farm fertility levels. Superphosphate (11 g lysimeter^{-1} equivalent to 555 kg ha^{-1}) and hydrated lime (20 g lysimeter^{-1} equivalent to 1 t ha^{-1}) was applied to each PRG/WC lysimeter and soil block. The fertiliser was sprinkled evenly over the lysimeters/soil blocks. The fertiliser was washed in to the soil by applying 4 L (20 mm) of water per lysimeter or soil block. All the lucerne lysimeters/soil blocks had 4 L of water applied, to ensure identical moisture inputs.

A second application of hydrated lime (20 g lysimeter^{-1} equivalent to 1 t ha^{-1}) was applied to the PRG/WC lysimeters/soil blocks on 18 September 2017. This lime was applied evenly over the lysimeters/soil blocks. The lime was applied during a rainfall event to ensure it was washed into the soil profile.

Table 6. Results of the basic soil analysis performed at the two lysimeter collection sites, at varying depths. Perennial ryegrass/white clover (PRG/WC) site (43°38'37.7"S 172°20'38.8"E) and lucerne site (43°38'51.8"S 172°21'01.0"E).

Crop type	Depth (mm)	pH	Olsen P (mg L^{-1})	Exchangeable Ca (me 100 g^{-1})	Exchangeable Mg (me 100 g^{-1})	Exchangeable K (me 100 g^{-1})	Exchangeable Na (me 100 g^{-1})
PRG/WC	**0-75**	6.0	15	6.6	1.0	0.6	0.2
PRG/WC	**0-100**	5.7	8	4.8	0.4	0.1	0.2
	100-200	5.5	7	4.2	0.2	0.1	0.2
	200-300	5.5	15	2.4	0.1	0.1	0.2
	300-450	5.4	17	0.8	<.05	<.05	0.1
	450-700	5.7	9	0.7	0.1	<.05	0.1
Lucerne	**0-75**	6.4	25	9.3	1.1	0.8	0.1
Lucerne	**0-100**	6.2	28	8.6	1.0	0.5	0.1
	100-200	5.9	38	8.7	0.8	0.2	0.2
	200-300	5.7	37	4.7	0.6	0.2	0.1
	300-450	5.7	23	2.7	0.4	0.2	0.1
	450-700	5.9	14	1.3	0.2	0.1	0.1

Experimental design

The experiment consisted of five treatments, with five replicates of each treatment (Table 7). There were two crop types; PRG/WC and lucerne. The C source that was readily available to microbes was sucrose, applied at two different rates (rate 1 = 12 t sucrose ha^{-1} and rate 2 = 24 t sucrose ha^{-1}) along with a no C treatment. C rate 2 was applied only to PRG/WC. All lysimeters/soil blocks received urine at a rate of 700 kg N ha^{-1}. The treatments were arranged in the trench facility at ADRDS using a randomised complete block design.

Table 7. Treatments applied to the lysimeters and soil blocks. Carbon (C) rate 1 = 12 t sucrose ha^{-1}. C rate 2 = 24 t sucrose ha^{-1}. Perennial ryegrass/white clover (PRG/WC).

Treatment number	Crop	Urine rate (kg N ha^{-1})	Carbon input	Replicates
1	PRG/WC	700	-	5
2	PRG/WC	700	C rate 1	5
3	PRG/WC	700	C rate 2	5
4	Lucerne	700	-	5
5	Lucerne	700	C rate 1	5

Treatments

Simulated grazing

On 30 June 2017, the PRG/WC lysimeters/soil blocks were trimmed to a residual mass equivalent of 1500 kg DM ha^{-1} using electric hand shears, to simulate winter grazing. The lucerne lysimeters/soil blocks did not need to undergo harvesting as they had very little late autumn/winter growth.

Simulated trampling of the lysimeters/soil blocks was performed on 3 July 2017. The soil in all lysimeters/soil blocks was trampled using cow hoof simulation equipment designed to provide approximately 200 kPa – similar to the pressure exerted by an adult cow hoof (Di *et al*. 2001). Each lysimeter/soil block received 6 'stomps' from the cow hoof.

Sucrose

The two rates of readily available C, 12 t sucrose ha^{-1} (equivalent to 5.1 t C ha^{-1}) and 24 t sucrose ha^{-1} (equivalent to 10.1 t C ha^{-1}), were applied according to the treatment design (Table 7). The sucrose was applied 2 July 2017; this allowed the microbial population time to consume the C (Johnson & Edwards 1979; Dalenberg & Jager 1981; Shepherd *et al*. 2010), before the urine was applied 4 July 2017. To apply the sucrose two master solutions were made. These solutions were made to concentrations (C rate 1 = 221.7 g sucrose L^{-1}. C rate 2 = 443.4 g sucrose L^{-1}) that allowed the intended rate of sucrose to be applied through the application of 1 L of solution to each lysimeter/soil block. This solution was applied using 2 x 500 mL bottles with rose head lids. Every lysimeter/soil block not receiving sucrose had 1 L of water applied, using an identical application method. This was performed to ensure the lysimeters/soil blocks received identical moisture inputs.

Urine collection and application

On 3 July 2017, urine was collected from 100 lactating Friesian/Jersey cross cows at the ADRDS. The cows' diet consisted of PRG/WC pasture (11 kg DM cow^{-1} day^{-1}) and PRG/WC baleage (4.5 kg cow^{-1} DM day^{-1}). Samples of the urine (50 mL) were taken and analysed for N concentration, using an Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany). The urine was stored overnight, in clean plastic barrels in a cool dark shed at the ADRDS. On 4 July 2017, after receiving the results from the urine N concentration analyses, the urine was standardised to 6.9 g N L^{-1} using 703.1 g of urea in 125 L of urine. Each lysimeter/soil block had 2 L (10 mm) of the urine applied on the 4 July 2017, at a rate equivalent to 700 kg N ha^{-1} using a rose head watering can, allowing an even spread of urine over the lysimeter/soil block.

Irrigation

A total of 470 mm of irrigation was applied to each lysimeter/soil block between 4 October 2017 and 3 April 2018. Irrigation was applied using a small scale pivot irrigation system at a rate of 5 to 10 mm per application. The irrigation scheduling simulated on farm practices to replace evapotranspiration losses.

Measurement methods

Leachate was collected from each lysimeter through plastic tubing attached to an outlet nozzle at the bottom of the lysimeters, leading to 10 L collection containers. Leachate was collected after each

drainage event. Leachate volumes were measured and 50 mL samples were taken for chemical analysis. Samples were kept frozen at -20 °C until they were analysed for ammonium (NH_4^+) and NO_3^- using a FOSS FIAstar 5000 twin channel analyser with SoFIA software version 2.00 (Gal *et al*. 2004). Leachate samples were also analysed for C content (Organic and Inorganic) using a Shimadzu Total Organic C analyser (TOC-5000A) fitted with a Shimadzu ASI-5000A autosampler.

Gas samples were collected using a closed chamber method similar to that described in Hutchinson and Mosier (1981). The gas chambers were constructed from a metal cylinder insulated with 25 mm thick polystyrene foam to avoid heating of the atmosphere in the chamber during sampling. The chamber was placed inside a small water trough which was mounted around the top of each lysimeter casing. The chamber was placed there for 40 minutes during which time three 20 mL gas samples were collected, 20 minutes apart. The gas samples were collected using a syringe through a rubber septum on top of the gas chamber. Samples were collected twice weekly for the first three months after urine application and then once weekly for the fourth month after urine application. The N_2O concentration of the gas samples was measured using a gas chromatograph (Model 8610C, SRI Instruments, California, USA) linked to a Gilson GX-271 autosampler (Gilson Inc, MI, USA).

The PRG/WC lysimeters were harvested once plant development had reached the 2-3 leaf stage and yields were on average 3000 kg DM ha^{-1}. The herbage was cut to a residual height of approximately 50 mm (1500 kg DM ha^{-1}). The lucerne was harvested according to seasonal management information in Moot *et al*. (2003), which resulted in six harvests. The pasture/lucerne was collected into paper bags. Dry matter production was determined gravimetrically after drying at 70 °C for 72 hours. The dry pasture/lucerne samples were then ground using a Retsch Ultra Centrifugal Mill ZM 200, with a 1 mm sieve and running at a speed of 18,000 rpm. The samples were stored in sealed 70 mL containers at room temperature in the dark, until analysis for their total N content using an Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany).

Soil samples were taken from the soil blocks to elucidate the mechanisms involved in the transformation of soil mineral N. Samples were taken using a soil corer (100 mm depth, 75 mm diameter) on days 0, 1, 7, 14, 28, 56 and 112 after urine application. Holes remaining after core removal were immediately back-filled with a soil-sand mix and identified using plastic markers to prevent subsequent samplings occurring from that same position. The samples were stored at -80°C until analysis. To determine the soil mineral N (NO_3^- and NH_4^+) a potassium chloride (KCl) extract was performed on 5 g of field moist soil. This was performed by adding 25 mL of 2M KCL extraction solution to the sample. The sample was then shaken for 1 h on an end over end shaker, centrifuged at 4000 rpm for 3 minutes and then filtered through 110 mm Munktell Grade 393 filter paper funnels.

The sample was frozen at -18 °C until the concentration of NH_4^+, NO_3^- could be determined on a Lachat QuikChem 8500 Series 2 Flow Injection Analysis System (Lachat Instruments, Loveland, CO, USA).

In agricultural soil, nitrification is largely driven by soil ammonia oxidising bacteria (AOB) and ammonia oxidising archaea are not involved (Di *et al*. 2009b). Therefore, DNA extraction and soil AOB ammonia monooxygenase (*amoA*) gene abundance was measured using quantitative polymerase chain reaction (qPCR), following methodologies adapted from Di *et al*. (2009b). DNA was extracted from the soil using a NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany) as per the manufacturer's instructions. In brief, a 0.25 g soil sample was taken and placed in a NucleoSpin® Bead tube. To this, 700 µL of Buffer SL2 and 250 µL of Enhancer SX were added. The sample was then homogenised for 1 min using a FastPrep®-24 Sample Preparation System (M.P. Biomedicals, California, USA) at a speed of 6 m s^{-1}. The sample was then centrifuged at 11,000 rpm for 2 min (Centrifuge 5424, Eppendorf AG, Hamburg, Germany). The supernatant was transferred into a sterilised 1.7 mL tube, to which a 150 µL of Buffer SL3 was added. The samples were then shaken for 5 s, incubated at 4°C for 5 min, then centrifuged at 11,000 rpm for 1 min. Up to 700 µL of supernatant was then transferred into a NucleoSpin® Inhibitor Removal Column fitted on top of a collection tube, and then centrifuged at 11,000 rpm for 1 min. The column was then discarded and 250 µL of Buffer SB was added to the flow through and mixed with a pipette. A 550 µL sample of this was pipetted onto a NucleoSpin® Soil Column on top of a clean collection tube. This was then centrifuged at 11,000 rpm for 1 min and the flow through was discarded. This step was repeated until no sample remained. Next, 500 µL of Buffer SB was added to the NuceloSpin® Soil Column and centrifuged for 30 s, with the flow through discarded. This process was then repeated with 550 µL of Buffer SW2, and then twice with 700 µL of Buffer SW2. After the final flow through was discarded, the column and collection tubes were then centrifuged at 11,000 rpm for 2 min, to ensure any residual ethanol was removed. The NuceloSpin® Soil Column was transferred to a new collection tube, to which 100 µL of Elution Buffer SE was added. The DNA was then eluted by incubating the samples at room temperature for 1 min and then centrifuging at 11,000 rpm for 30 s. DNA was then stored at -80 °C for further analysis.

AOB *amoA* gene abundance was measured using real time qPCR on a Rotor-Gene™ 6000 (Corbett Research, Australia). All qPCR reactions were prepared using a CAS1200 Robotic liquid handling system (Corbett Robotics, Australia). The AOB *amoA* genes were quantified using the primer pairs amoA-1F (5'-GGGGHTTYTACTGGTGGT-3') and amoA R-i (5'-CCCCTCNGNAAANCCTTCTTC-3') (Hornek *et al*. 2006). A 16 µL reaction mixture contained 8 µL 2x SYBR® Premix Ex Taq™ (Tli RNaseH Plus, Takara Bio Inc., Shiga, Japan), 0.4 µL of each prime, and sterile deionised water to bring up to a total of 14.5 µL and 1.5 µL of DNA sample. The DNA samples were diluted 10 times with deionised water prior to use. Serial dilutions of standards with a range of 10^1 to 10^7 copies μL^{-1} were run in duplicate to produce standard

curves. Once the PCR reactions were prepared the RotorDisc™ 100 was sealed using a Gene-Disc™ Heat Sealer (HS-01, Corbett Research, Australia). The qPCR temperature profiles used are given in Table 8. After amplification a melting curve analysis was performed to check for nonspecific amplification products. The florescence was measured continuously as the temperature gradually increased from 72 °C to 99 °C. Data were then analysed using the Rotor-Gene™ series software 1.7.

Standard curves for real time qPCR were created using the following process: Bacterial *amoA* gene was amplified from the extracted DNA using the previously mentioned primers. A PCR clean up kit (Axygen) was then used to purify the PCR products which were then cloned into the pGEM-T Easy Vector (Promega, Madison, WI). Following the manufacturers' instructions, the resulting clones were transformed in *Escherichia coli* JM109 competent cells (Promega). The transformed *E.coli* cells were grown on solid LB plates at 37 °C overnight. Ten to fifteen bacterial colonies from the plate were then individually inoculated into a 3 mL LB broth medium and incubated overnight in an orbital incubator – shaker at 37 °C and 250 rpm. Using a QIA Prep spin Miniprep Kit (Qiagen, Crawley, UK) the plasmids carrying correct the gene inserts were extracted. The DNA concentration was determined on a Qubit™ Fluorometer (Invitrogen™, New Zealand). The copy number of target genes were then calculated directly from the concentration of purified DNA. To produce an external standard curve, tenfold serial dilutions of a known copy number of the PCR amplifications were then subjected to real-time PCR assay in duplicate.

Table 8. Cycling conditions for PCR reactions (Di *et al*. 2009b).

Number of cycles	Cycling conditions	Temperature (°C)	Time (s)
1	Initial denaturation	94	120
40	Denaturation	94	20
	Primer annealing	57	30
	Extension	72	30

Statistical analysis

The data sets were subject to analysis of variance (ANOVA) using Genstat (18th edition, VSN International Ltd.). The C leaching data set was log transformed to ensure homogeneity of residual errors. For each ANOVA, orthogonal contrasts (2 x 2 factorial) were used to determine the significance of the main effect of crop type (lucerne vs PRG/WC) and of C application (no C input vs C rate 1) and their interaction (crop x C application). An additional contrast was used to determine if there was an effect of increasing the C rate for PRG/WC, from C rate 1 to C rate 2.

Results

Climate conditions and water inputs

Over the experimental period (4 July 2017 - 3 July 2018), the average daily air temperature ranged from a high of 23.7 °C in December 2017 to a low of 0.8 °C in June 2018 (Figure 19a). Daily average soil temperature (100 mm depth) ranged from a low of 3.4 °C in July 2017 to a high of 27.9 °C in December 2017 (Figure 19a). Water inputs over the 12 month experimental period totalled 1363 mm, comprising 877 mm of rainfall, 470 mm of irrigation and 16 mm of water input from treatments (Figure 19b). The 12 month period had higher rainfall than the average (618 mm), due to a very wet winter in 2017 (Macara 2016).

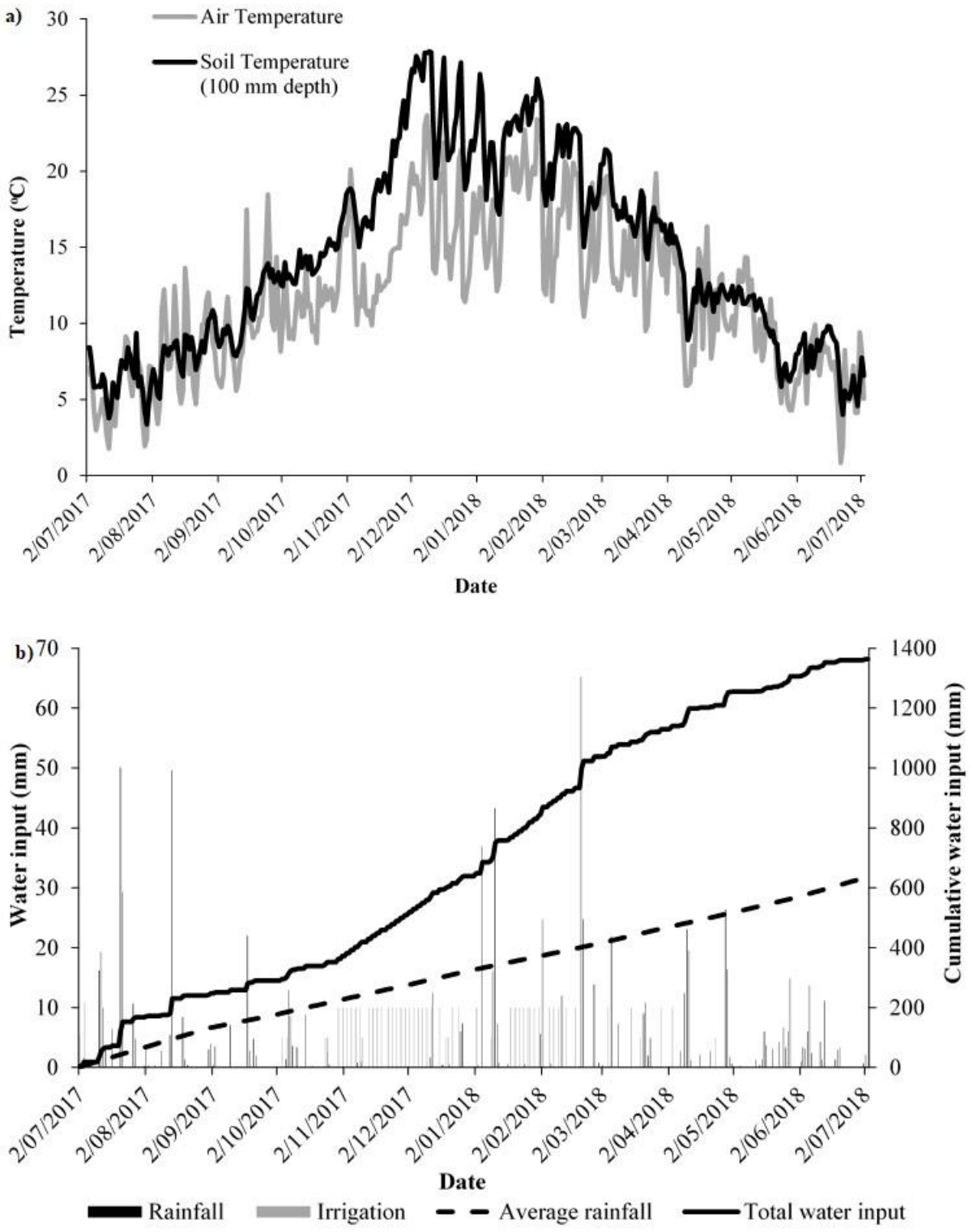

Figure 19. a) Average daily air temperature and soil temperature (at 100 mm), and b) daily rainfall, irrigation water inputs and cumulative water input over the experimental period (4 July 2017 - 3 July 2018), and long-term average rainfall data (1971-2000, Lincoln weather station 4881).

Leaching losses

Nitrate leaching losses

The effect of applying readily available C on the NO_3^--N concentration in the leachate and the total amount of NO_3^--N leached, under PRG/WC pasture with urine applied is shown in Figure 20. A complete NO_3^- leaching breakthrough curve under the PRG/WC is shown in Figure 20a, with NO_3^- concentrations peaking at 55.9, 22.3 and 5.5 mg NO_3^--N L^{-1} under the no C input, C rate 1 (12 t sucrose ha^{-1}) and C rate 2 (24 t sucrose ha^{-1}) treatments, respectively. These NO_3^- concentration peaks occur between 230-270 mm of cumulative drainage. The application of C rate 1 (12 t sucrose ha^{-1}) and C rate 2 (24 t sucrose ha^{-1}) significantly reduced the amount of NO_3^--N leaching by 75% and 91%, respectively (Figure 20b).

The effect of C application on NO_3^--N concentration and total NO_3^--N leached under lucerne with urine applied, is shown in Figure 21. A complete NO_3^- leaching breakthrough curve under the lucerne is shown in Figure 21a, with NO_3^- concentrations peaking at 175.2 and 121.6 NO_3^--N L^{-1} for no C input and C rate 1 treatments, respectively. These NO_3^- concentration peaks occur at 270 mm of cumulative drainage**.** The application of C rate 1 significantly decreased (51%) total NO_3^--N leaching (Figure 21b).

Total leaching losses

The effects of each treatment on total NO_3^--N, NH_4^+-N, mineral N, log transformed carbon leached over the sampling period (4 July 2017- 17 May 2018) and the total amount of annual drainage (4 July 2017-3 July 2018) are shown in Table 9. There was a significant effect of crop type ($P < 0.001$) and C rate 1 ($P = 0.039$) on NH_4^+-N leaching. Lucerne had greater NH_4^+-N leaching loss compared with PRG/WC and the application of C rate 1 decreased NH_4^+-N leaching. However, the total amount of NH_4^+-N leached under the C rate 2 treatment was not significantly different from the other two PRG/WC treatments (no C input and C rate 1). There was a significant effect of crop type ($P < 0.001$) and C rate 1 ($P < 0.001$) on mineral N leaching. Lucerne had greater mineral N leaching loss compared with PRG/WC and the application of C at rate 1 decreased mineral N leaching. The application of C was shown to significantly increase the amount of total C leached ($P < 0.001$). Crop type had no significant effect on total C leached ($P = 0.098$). There was no significant difference in total annual drainage among the treatments.

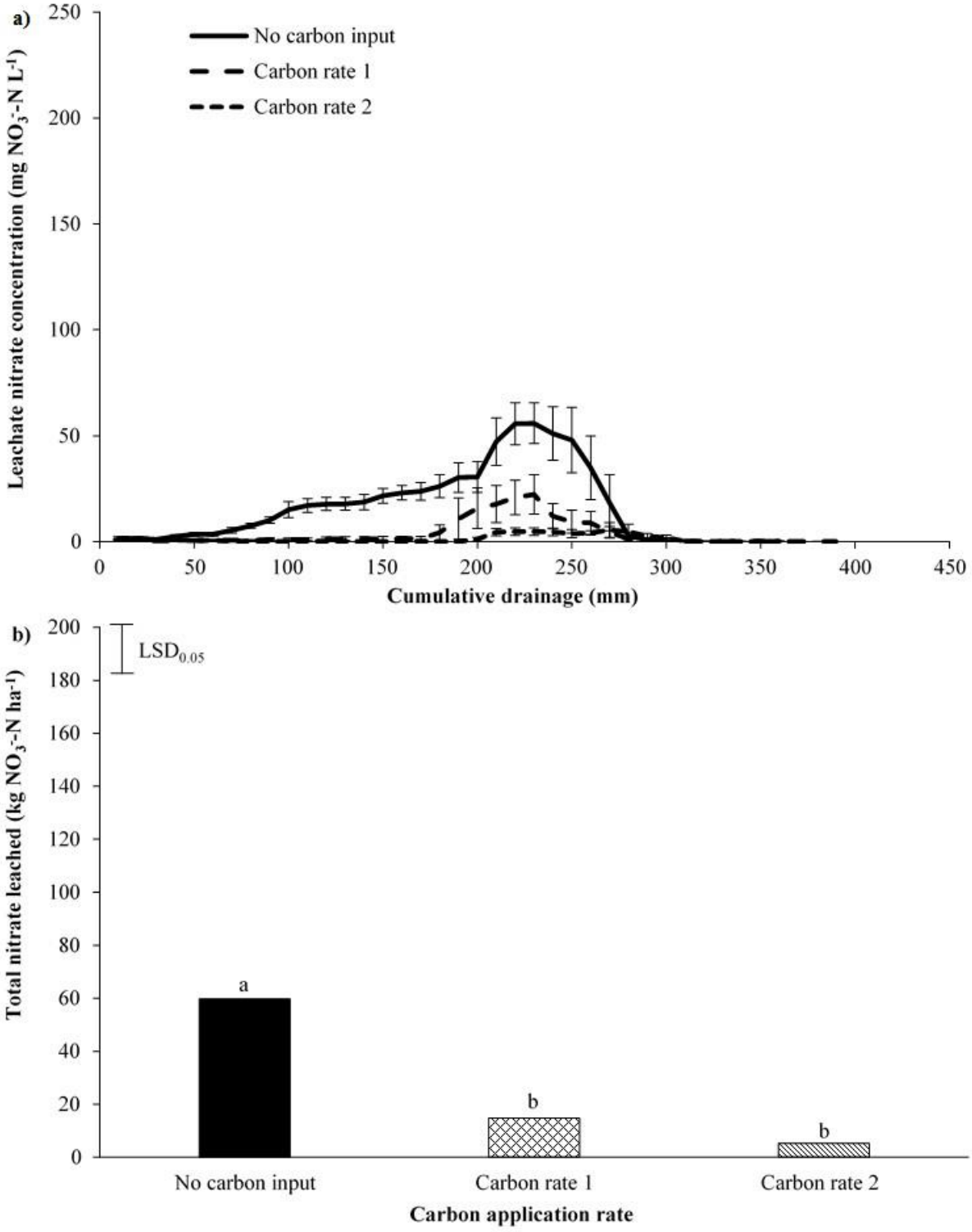

Figure 20. a) The average nitrate concentration in leachate plotted against cumulative drainage (mm) and b) total nitrate leached over the sampling period (4 July 2017- 17 May 2018), under ryegrass/white clover pasture. a) Error bars are standard error of the mean (*n* = 5). b) Least significant difference (LSD) is at the 5% level (*n* = 5). LSD = 18.9. Bars with a letter in common are not significantly different at the 5% level.

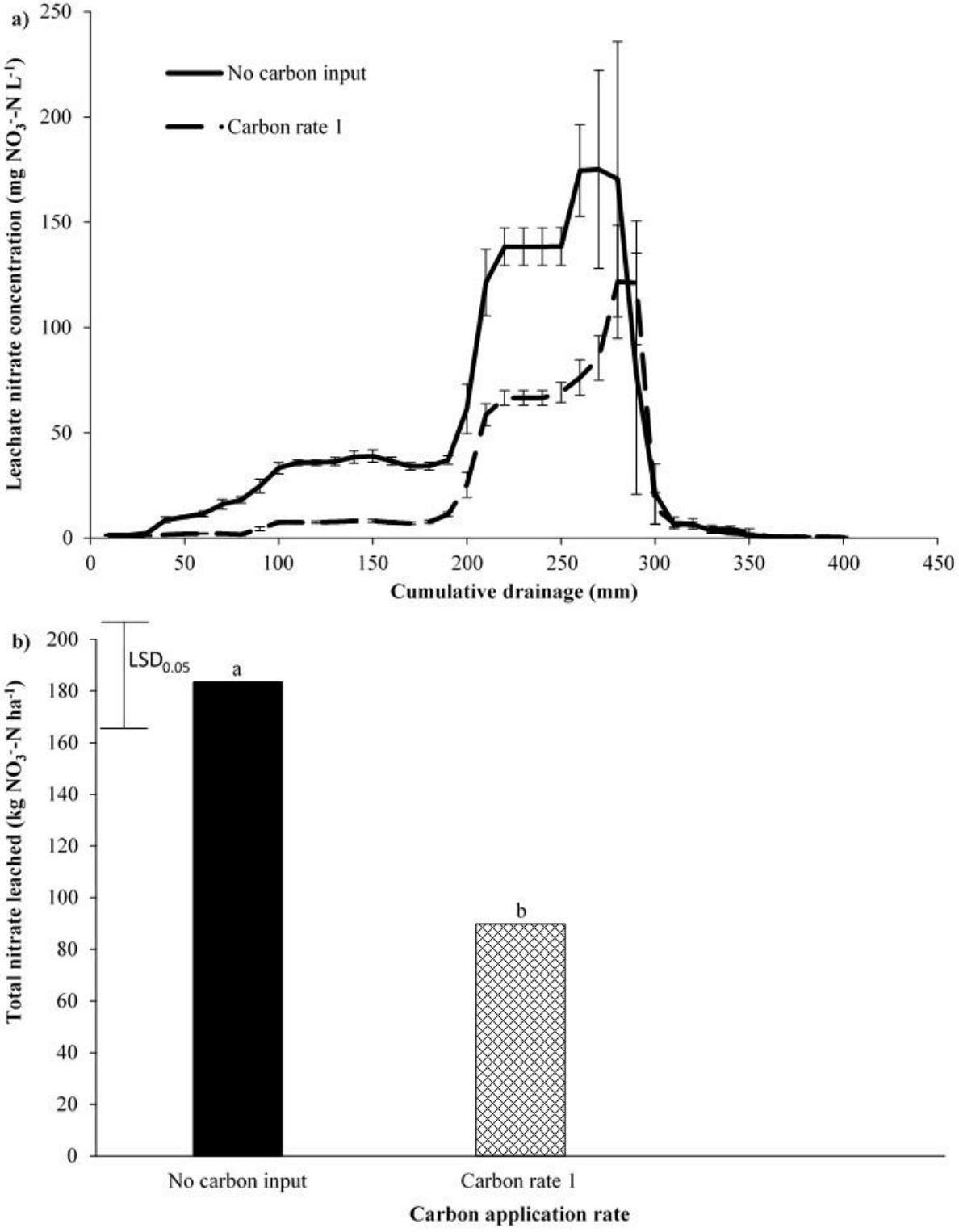

Figure 21. a) The average nitrate concentration in leachate plotted against cumulative drainage (mm) and b) total nitrate leached over the sampling period (4 July 2017- 17 May 2018) under lucerne. a) Error bars are standard error of the mean (*n* = 5). b) Least significant difference (LSD) is at the 5% level (*n* = 5). LSD = 46.2. Bars with a letter in common are not significantly different at the 5% level.

Table 9. The effect each treatment had on total nitrate, ammonium, mineral N and log transformed total carbon leached (brackets contain back transformed means) over the sampling period (4 July 2017- 17 May 2018) and the total amount of annual drainage (4 July 2017- 3 July 2018). The first three contrasts are main effect (m.e.) and interaction contrasts for a 2 x 2 factorial, with factors crop type (perennial ryegrass/white clover (PRG/WC), lucerne) and sucrose rate (no carbon, rate 1). The fourth contrast is between PRG/WC rate 1 and rate 2.

Crop	Carbon rate	Nitrate leached (kg NO_3^--N ha^{-1})	Ammonium leached (kg NH_4^+-N ha^{-1})	Mineral N leached (kg N ha^{-1})	Drainage volume (mm)	Log carbon leached (Back transformed data) (kg C ha^{-1})
PRG/WC	No C	59.8	70.1	129.9	488	2.304 (201)
	C rate 1	14.8	54.5	69.4	493	2.935 (861)
	C rate 2	5.3	72.0	77.2	467	3.316 (2070)
Lucerne	No C	183.4	128.6	312.0	477	2.548 (353)
	C rate 1	89.8	93.7	183.5	498	2.907 (807)
LSD (5%) (*n*=5)		24.3	33.7	38.4	64	0.185
P values for contrasts	Crop m.e.	<0.001	<0.001	<0.001	0.887	0.098
	No carbon vs C rate 1 m.e.	<0.001	0.039	<0.001	0.549	<0.001
	Interaction: Crop x C rate	0.009	0.399	0.018	0.703	0.043
	PRG/WC C rate 1 vs rate 2	0.411	0.286	0.667	0.407	<0.001

Nitrous oxide emissions

The effect of each treatment on total N_2O-N emissions over the sampling period are shown in Figure 22. There was a significant (*P* = 0.04) main effect of crop type (PRG/WC vs lucerne), with the PRG/WC having significantly higher N_2O emissions than lucerne. The application of sucrose appears to have slightly increased N_2O emissions, however, there was no significant main effect of C application (no C input vs C rate 1) (*P* = 0.205) on N_2O-N emissions. There was no significant interaction between crop x C application (*P* = 0.297). For PRG/WC, there was no significant difference in N_2O-N emissions between C rate 1 and 2 (*P* = 0.596).

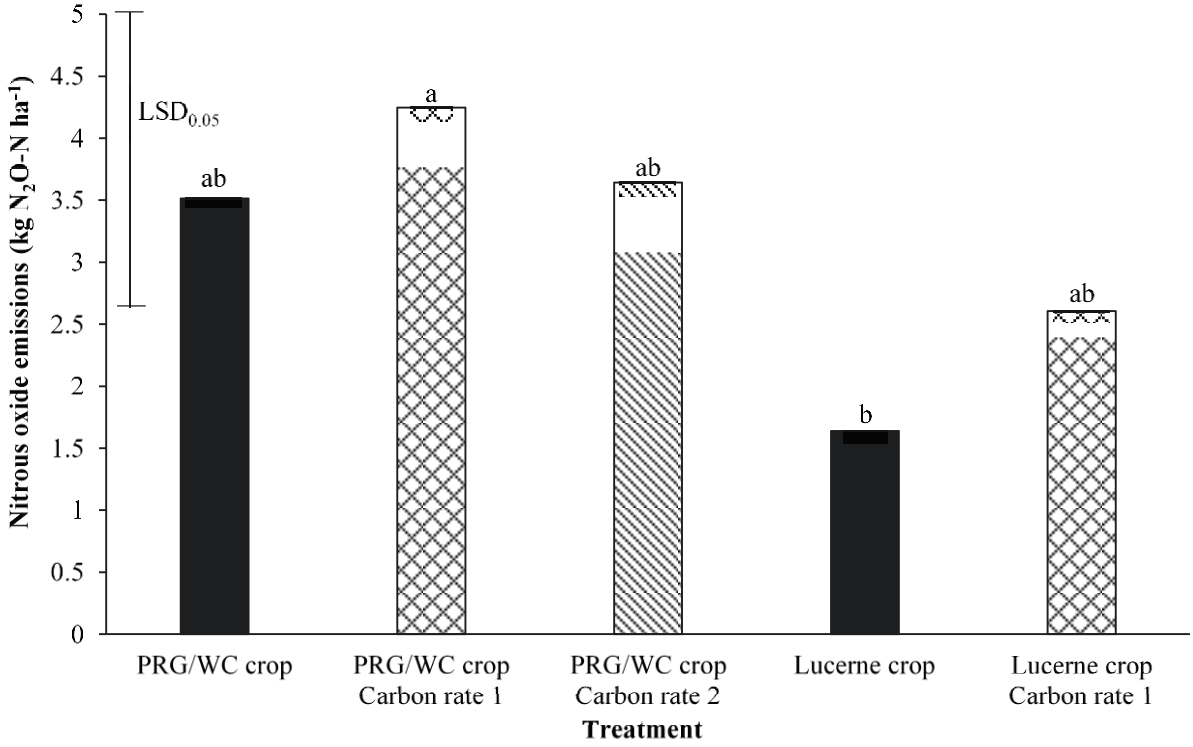

Figure 22. The effect of different treatments on nitrous oxide emissions over the sampling period (4 July - 31 October). Least significant difference (LSD) is at the 5% level (*n* = 5). LSD = 2.36. Bars with a letter in common are not significantly different at the 5% level.

Herbage yield and nitrogen uptake

Yield

The application of C had no signifcant effect on PRG/WC yield (Figure 23a), with the PRG/WC pasture treatments producing annual DM yields between 15,095 and 16,253 kg DM ha^{-1}. However, the application of C signifcantly (*P* = 0.008) reduced lucerne yield by 19% (Figure 23b). The lucerne yield dropped from 8,961 kg DM ha^{-1} under the no C input, to 7,222 kg DM ha^{-1} under the C rate 1 treatment.

Plant nitrogen uptake

The application of C had no signifcant effect on total N uptake by the PRG/WC (Figure 24a), with the PRG/WC pasture treatments having an annual plant N uptake of between 480.3 and 515.9 kg N ha^{-1}. However, the application of C signifcantly (*P* = 0.011) reduced total N uptake by the lucerne N by 17% (Figure 24b). The lucerne plant N uptake dropped from 256.2 kg N ha^{-1} under the no C input, to 212.1 kg N ha^{-1} under the C rate 1 treatment.

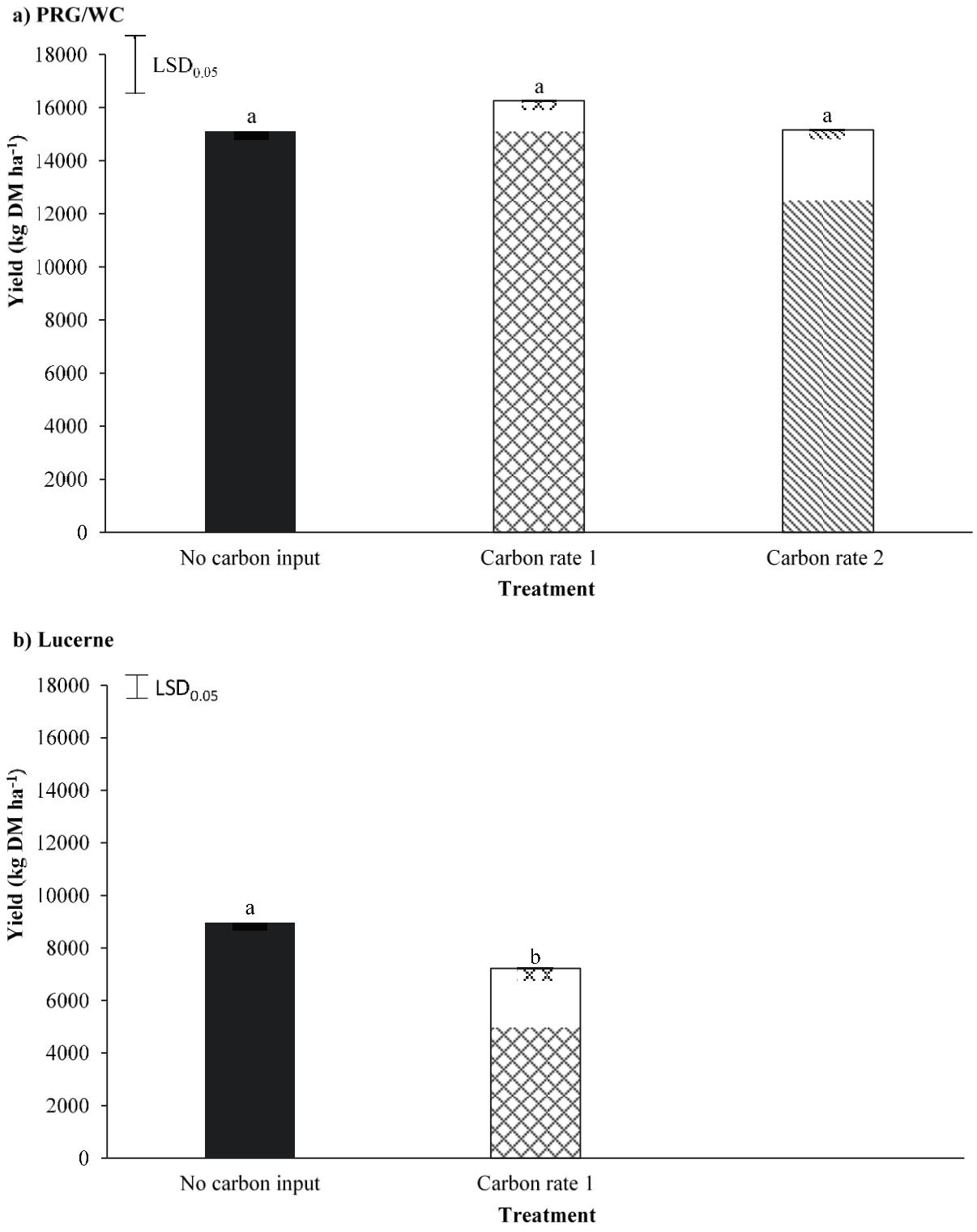

Figure 23. a) The effect carbon application (Carbon rate 1 = 12 t sucrose ha^{-1}; Carbon rate 2 = 24 t sucrose ha^{-1}) had on annual perennial ryegrass/white clover (PRG/WC) yield and b) the effect carbon application had on annual lucerne yield. Least significant difference (LSD) is at the 5% level (*n* = 5). PRG/WC LSD = 2130. Lucerne LSD = 974. Bars with a letter in common are not significantly different at the 5% level.

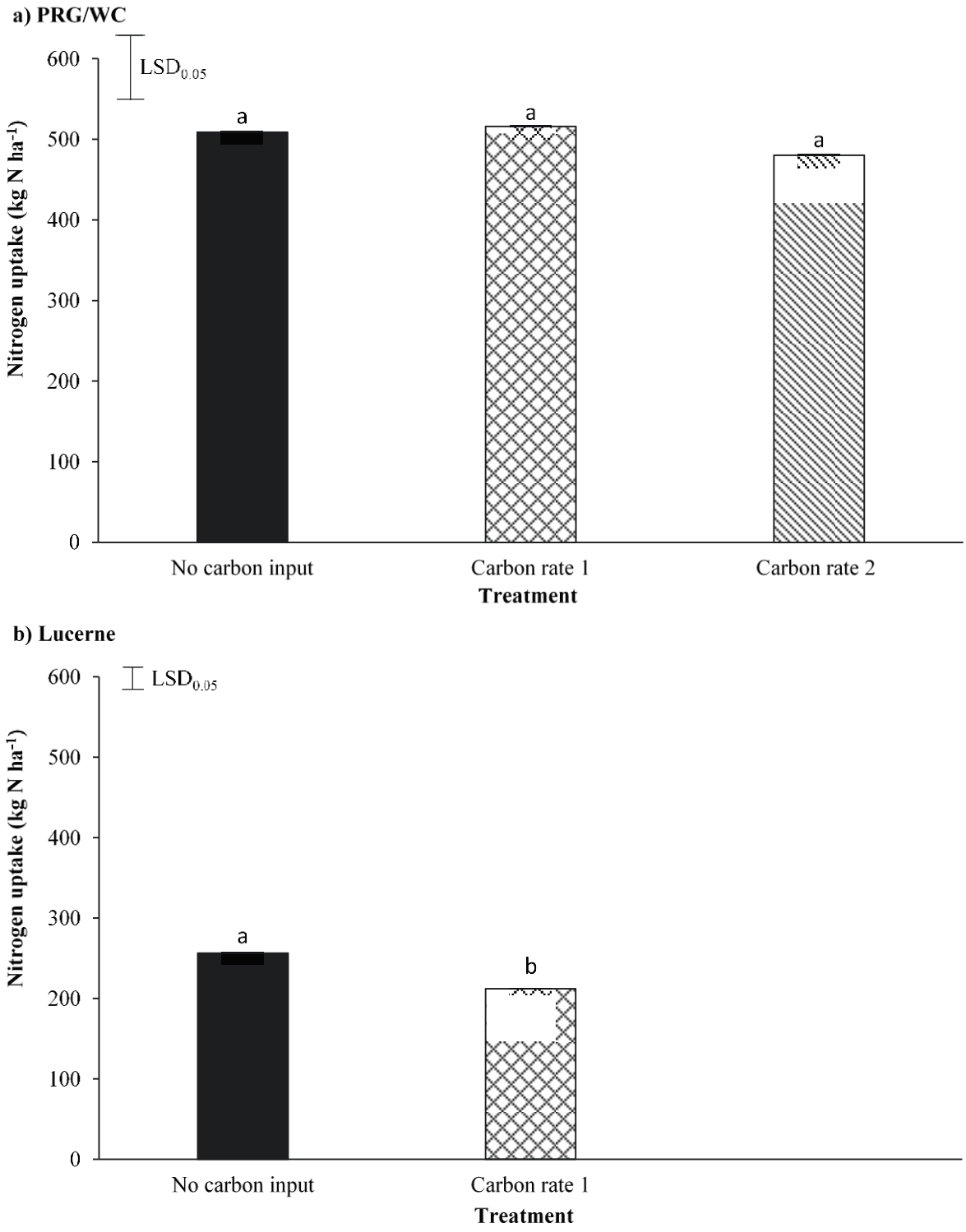

Figure 24. a) The effect carbon application (Carbon rate 1 = 12 t sucrose ha^{-1}; Carbon rate 2 = 24 t sucrose ha^{-1}) had on perennial ryegrass/white clover (PRG/WC) total annual nitrogen (N) uptake and b) the effect sucrose application had on lucerne total annual N uptake. Least significant difference (LSD) is at the 5% level (*n* = 5). PRG/WC LSD = 79.1. Lucerne LSD = 26.8. Bars with a letter in common are not significantly different at the 5% level.

Soils

Mineral N

The effect of the C applications on total soil mineral N (NO_3^--N + NH_4^+-N), over the first 112 days of the sampling period, under PRG/WC and lucerne crops is shown in Figure 25a and b. The large increase in soil mineral N from day 0 to day 1 is due to the application of urinary-N. Thereafter, a continual decrease in soil mineral N was observed, under both PRG/WC and lucerne. Averaging over the period from day 1 to 28 after urine application using the trapezoid rule to calculate the area under the curve (AUC), the application of C significantly reduced the total amount of soil mineral N under both PRG/WC (P = 0.002) and lucerne (P = 0.001).

AOB gene abundance

The effect of different rates of C application on soil AOB *amoA* gene abundance under PRG/WC and lucerne, over the first 112 days after urine application is shown in Figure 26a and b. After day 14, the AOB population was consistently lower for the C treatments compared to the control treatment for both PRG/WC and lucerne. However, the AOB population was not affected by the rate of C addition (Figure 26a). Using the AUC for the first 112 days after urine application, the C treatments had significantly lower AOB *amoA* gene abundance, under both PRG/WC (P = 0.001) and lucerne (P = 0.009).

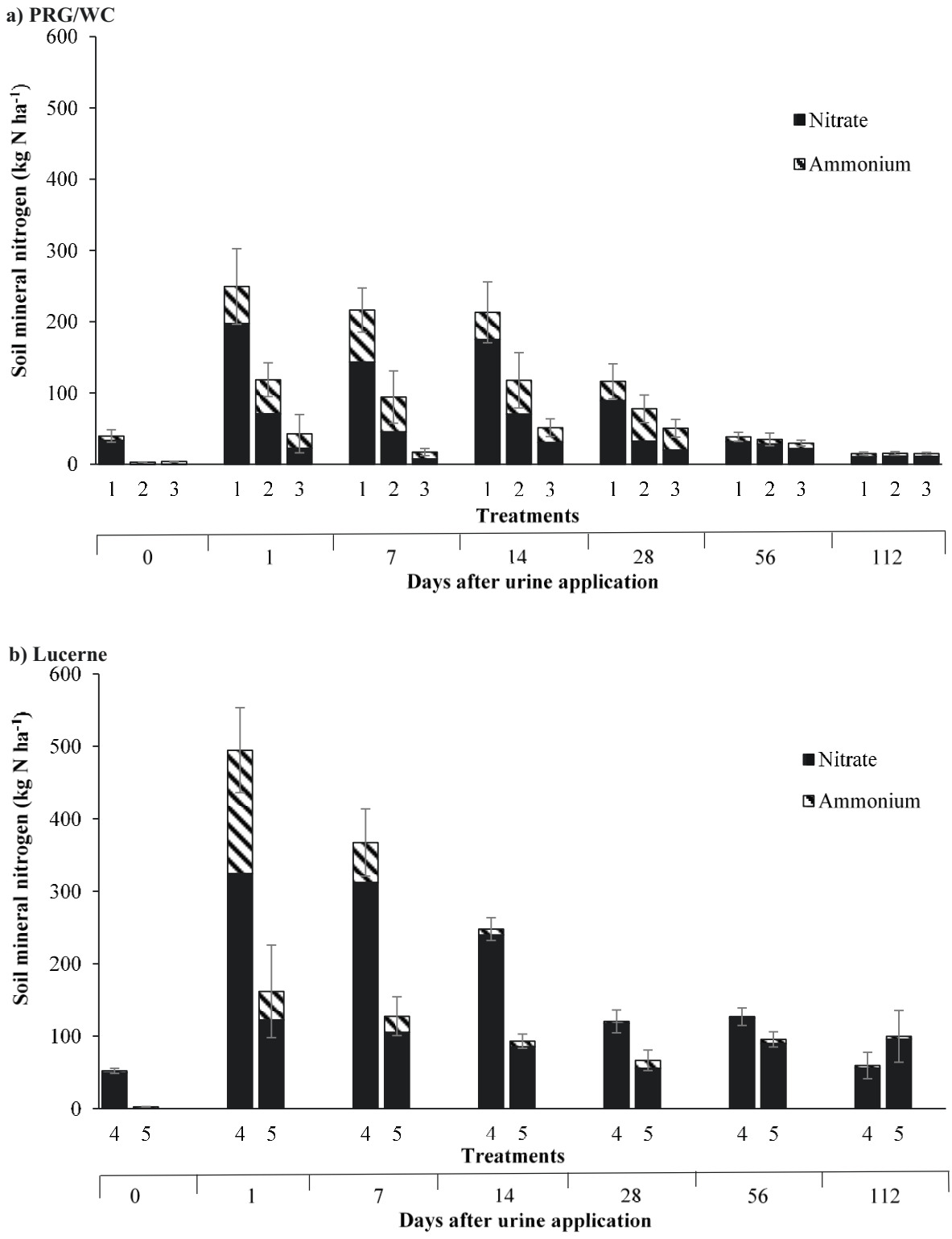

Figure 25. The effect different rates of carbon application (Carbon rate 1=12 t sucrose ha^{-1}; Carbon rate 2=24 t sucrose ha^{-1}) had on the soil mineral nitrogen (NO_3^--N + NH_4^+-N) over the sampling period (4 July 2017- 24 October 2017), under a) perennial ryegrass/white clover (PRG/WC) and b) lucerne. Soil samples were taken from 0-100 mm depth. Treatments: 1= PRG/WC crop, no carbon input. 2= PRG/WC crop, carbon rate 1. 3= PRG/WC crop, carbon rate 2. 4= Lucerne crop, no carbon input. 5= Lucerne crop, carbon rate 1. Error bars are standard error of the mean for total mineral nitrogen (*n* = 5).

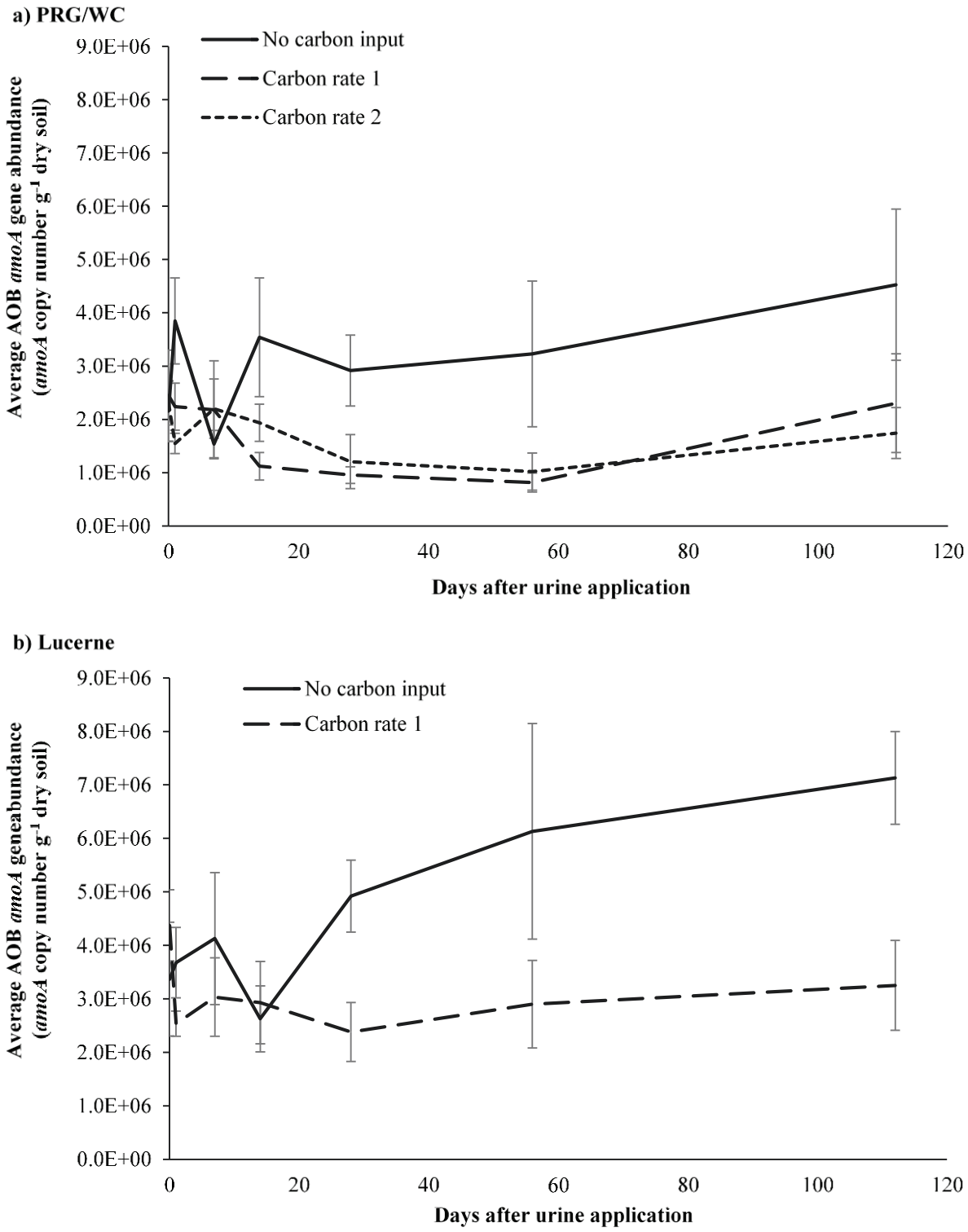

Figure 26 . The effect carbon application (Carbon rate 1=12 t sucrose ha^{-1}; Carbon rate 2=24 t sucrose ha^{-1}) had on soil AOB *amoA* gene copies in A) perennial ryegrass/white clover (PRG/WC) soil and B) lucerne soil, over the first 112 days after urine application. Samples were taken from 0-100 mm depth in the soil profile. Error bars are standard error of the mean (*n* = 5).

Discussion

In this experiment the application of C (sucrose) was shown to reduce the amount of soil mineral N (both NO_3^--N and NH_4^+-N) under both PRG/WC and lucerne (Figure 25a and b). This is attributed to immobilisation of N, especially in the C treated soil. This is consistent with literature that suggests that adding a C source that is readily available to microbes, increases immobilisation of mineral N (Johnson & Edwards 1979; Vinten *et al*. 2002; Rahn *et al*. 2003; De Neve *et al*. 2004; Eschen *et al*. 2007; Szili-Kovács *et al*. 2007; Rahn *et al*. 2009; Shepherd *et al*. 2010). The results of this experiment also showed that there was an inhibition of the AOB population growth when C was applied, under both PRG/WC and lucerne crops, compared with the no C input treatments (Figure 26a and b). The inhibition of AOB population growth was likely due to the decrease in the amount of soil mineral NH_4^+-N available to the microbes, under the C applied treatments (Figure 25a and b). AOB are autotrophic bacteria and obtain their energy from the oxidation of NH_4^+ (Kowalchuk & Stephen 2001; Norton *et al*. 2002). The reduction in the amount of NH_4^+-N due to immobilisation, the AOB's energy source, would likely have limited the AOB's population growth in the C treated soil. This lower amount of NH_4^+-N and the limitation of AOB population growth reduced the amount of NO_3^--N produced, compared with the non C treatments.

The addition of readily available C to soil reduced the amount of NO_3^--N leaching under the urine treatments (Figure 20 and 21). This reduction in NO_3^--N leaching loss was observed under both lucerne and PRG/WC treatments. There was a 51% decrease in NO_3^--N leaching loss when C was applied at rate 1 under lucerne (Figure 21). Under the PRG/WC pasture, C rate 1 (12 t sucrose ha^{-1}) and C rate 2 (24 t sucrose ha^{-1}) reduced NO_3^--N leaching by 75% and 91%, respectively (Figure 20). These reductions in NO_3^--N leaching loss are slightly higher than those reported by Shepherd *et al*. (2010), who reported reductions of 44% and 82% under C application rates of 12 t sucrose ha^{-1} and 24 t sucrose ha^{-1}, respectively. The greater reductions in NO_3^--N leaching losses from this study compared with Shepherd *et al*. (2010) may be due to the timing of C application relative to the urine application. In this study, the C was applied 2 days before urine application, allowing the microbial population time to consume some of the C before urine application. In contrast, in Shepherd *et al*. (2010) the C was applied immediately after urine application.

The application of C rate 1 to soil also reduced the amount of NH_4^+-N leached under the urine treated soil (P = 0.039), with the application of C rate 1 reducing NH_4^+-N leaching by 22% and 27% under PRG/WC and lucerne, respectively. However, the total amount of NH_4^+-N leached under the C rate 2 treatment was not significantly different from the other two PRG/WC treatments (no C input and C rate 1). The relatively large amounts of NH_4^+-N leached in this experiment were attributed to the shallow stony soil type and the high levels of rainfall and drainage occurring after urine application (Carey *et al*. 2017).

The reductions in the amounts of NO_3^--N and NH_4^+-N leached under the C applications were attributed to the increased biological immobilisation of N in the soil. This decrease in NO_3^--N and NH_4^+-N availability (Figure 25a and b), due to increased immobilisation, removed mineral N from the soil solution resulting in less mineral N being susceptible to leaching (Rahn *et al*. 2009; Shepherd *et al*. 2010).

The application of C significantly reduced total annual dry matter yield (Figure 23b) and N uptake (Figure 24b) by the lucerne. This reduction in growth and N uptake was attributed to the reduction in the amount of soil mineral N as a result of immobilisation (Figure 25a and b). However, under the PRG/WC crop there was no significant difference in total annual dry matter yield (Figure 23a) and N uptake (Figure 24a) among the C treatments. This lack of an effect of added C in the PRG/WC compared with the lucerne was attributed to different plant characteristics such as: greater autumn and winter activity of the PRG/WC which captured more N from the urine treatment than the lucerne, and a more fibrous root system (Baars *et al*. 1975; Evans 1978; McKenzie *et al*. 1990).

The PRG/WC treatments leached significantly less mineral N (both NO_3^--N and NH_4^+-N) than the lucerne treatments ($P < 0.001$). This is attributed to higher autumn and winter plant growth rates of the PRG/WC compared to lucerne (Baars *et al*. 1975; Woods *et al*. 2016), with lucerne having negligible winter growth (Teixeira 2006). This is again consistent with literature which highlights the importance of winter growth on reducing N leaching (Crush *et al*. 2005; Teixeira 2006; Nichols & Crush 2007; Popay & Crush 2010; Moir *et al*. 2013; Malcolm *et al*. 2014; Malcolm *et al*. 2015b; Carey *et al*. 2017).

The main factor affecting N_2O-N emissions in this trial was the crop type, with the PRG/WC treatments emitting more N_2O-N than the lucerne treatments ($P = 0.04$). The lower N_2O-N emission under the lucerne is attributed to the smaller amount of mineral N in the top soil under lucerne because of the large leaching loss that occurred. This increase in N leaching reduced the N available in the soil that can undergo denitrification and subsequently be lost as N_2O-N.

In contrast to previous studies (Bremner & Shaw 1958; Burford & Bremner 1975), the application of C was found to have no significant effect on N_2O-N emissions ($P = 0.205$). The addition of C to soil is thought to provide the organic C needed by soil denitrifiers for growth and respiration. However, due to the high rates of immobilisation of N under the C applied treatments in this study, there was less soil NO_3^--N available to denitrify (Figure 25). However, the lower N_2O-N emissions in the C treatments might also be due to enhanced complete denitrification to N_2.

This study has established that the application of readily available C can have significant effects on N transformations and consequently reduce N leaching losses from soil under urine patches, while not

significantly increasing N_2O emissions. With plants potentially releasing nearly 25% of the C they fix photosynthetically into the rhizosphere (Marschner 1995; Walker *et al*. 2003; Stockmann *et al*. 2013), there is an opportunity for further work to be done on using C inputs from plants to reduce N losses. Identifying plants that sequester C or release important C based compounds (e.g. biological nitrification inhibitors) could be a viable area of research that could result in the tightening of the N cycling in farm systems.

Conclusions

The main conclusion of the research was that adding readily available C to soil can significantly reduce N leaching losses without causing an increase in N_2O emissions. This was attributed to the added carbon increasing immobilisation of N in the soil.

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4.2.2 Additional information

^{15}N tracer

Introduction

A ^{15}N tracer was applied via the urine in lysimeter experiment 1 (Talbot *et al*. 2019; Talbot *et al*. 2020). However, Talbot *et al*. (2019) did not publish the ^{15}N tracer results. Section 4.2.2 will provide ^{15}N tracer results from the experiment described by Talbot *et al*. (2019).

Method

Urea enriched with ^{15}N (97.6 atom%) was added to the urine, this enriching the urine to 10 atom% ^{15}N. The ^{15}N enrichment additions to urine are shown in Table 10.

Table 10. Urine nitrogen concentrations, urea-N addition and ^{15}N enrichment values.

Original concentration	
Nitrogen (N) concentration (g N L^{-1})	4.6
Standardisation of N and ^{15}N enrichment	
Enrichment ^{15}N%	10
Mass of ^{15}N enriched urea-N used (g)	89.3
Mass of standard urea-N added (g)	239.1
Final concentrations after urea addition	
Final urine N concentration (g N L^{-1})	6.9
N application rate (kg N ha^{-1})	702.8

Herbage, leachate and N_2O emissions samples containing the ^{15}N tracer were collected and analysed as described in Section 3.2.4. Recoveries of ^{15}N (%) in herbage, leachate and N_2O emissions were calculated using the equation from Cabrera and Kissel (1989). A value of 0.3663% was used as the B value (atom % ^{15}N natural abundance enrichment). Interpolation was used to estimate the ^{15}N enrichment for those sampling occasions when ^{15}N analyses were not performed. For N_2O emissions, the ^{15}N recovery at each sampling date was integrated to estimate the total ^{15}N recovery during the sampling period.

Results

For this experiment, the effect each treatment had on the recovery % of the ^{15}N applied among the herbage, leachate and N_2O emission is shown in Table 11.

Table 11. Recovery (%) of ^{15}N (applied with the urine) in the herbage, leachate, and nitrous oxide fractions. Values with a superscript letter in common are not significantly different at the 5% level. The first three contrasts are main effect (m.e.) and interaction contrasts for a 2 x 2 factorial, with factors crop type and sucrose rate. The fourth contrast is between perennial ryegrass/white clover (PRG/WC) rate 1 and 2.

Crop	Carbon rate	Herbage	Leachate	Nitrous oxide emissions
PRG/WC	No C	36.5^{a}	14.5^{c}	0.3^{ab}
	C rate 1	30.9^{a}	7.6^{d}	0.4^{a}
	C rate 2	25.3^{b}	8.7^{d}	0.3^{ab}
Lucerne	No C	13.4^{c}	35.0^{a}	0.1^{b}
	C rate 1	11.5^{c}	20.4^{b}	0.2^{ab}
LSD (5%) (n = 5)		5.1	4.8	0.2
P values for contrasts	Crop m.e.	<0.001	<0.001	0.052
	No C vs C rate 1 m.e.	0.040	<0.001	0.238
	Interaction: Crop x C rate 1	0.293	0.028	0.968
	PRG/WC C rate 1 vs rate 2	0.033	0.626	0.376

There was a highly significant crop effect on ^{15}N herbage recovery ($P < 0.001$), with the PRG/WC crop having significantly higher ^{15}N herbage recovery than that of the lucerne. There was also a significant crop effect on ^{15}N leachate recovery ($P < 0.001$), with the lucerne crop having significantly higher ^{15}N leachate recovery than that for the PRG/WC crop. There was no significant crop effect (P =0.052) on ^{15}N N_2O emission recovery.

The application of C significantly reduced ^{15}N herbage recovery ($P = 0.040$) and ^{15}N leachate recovery ($P < 0.001$). The application of C had no effect on ^{15}N N_2O emissions recovery ($P = 0.238$).

Discussion

The results from the ^{15}N analysis strengthen the findings of Talbot *et al*. (2019). The higher recovery of ^{15}N tracer in the PRG/WC herbage compared with lucerne herbage, supports the findings of Talbot *et al*. (2019) that PRG/WC had higher winter plant growth; which subsequently resulted in greater uptake of the urine-N. The higher plant urine-N uptake by the PRG/WC herbage resulted in less urine-N

available to be leached. This is supported by the lower recovery of ^{15}N tracer in PRG/WC leachate compared with the lucerne leachate.

In the experiment the PRG/WC had higher N_2O emissions than the lucerne (P = 0.004); however, the recovery of ^{15}N tracer in N_2O was only borderline significantly higher for the PRG/WC (P = 0.052).

The results from the ^{15}N analysis strengthen the main conclusion found in Talbot *et al*. (2019). The lower recovery of ^{15}N tracer in both the herbage and leachate when C was applied, supports the theory that the application of C stimulated microbial populations, subsequently immobilising the N, making it temporarily unavailable for leaching or uptake by plants.

4.3 Trial 2 (Talbot *et al*. 2020)

4.3.1 Research article

This section has been published in Nutrient Cycling in Agroecosystems, published by Springer.

Cattle diet and winter plant growth effects on nitrogen losses from cattle urine patches

Abstract

Nitrogen (N) losses from agricultural land is a major environmental concern, as N leaching can cause eutrophication and nitrous oxide (N_2O) is a greenhouse gas. A lysimeter experiment was undertaken to investigate the effects of cattle diet and winter plant growth on N losses from cattle urine patches. The experiment was conducted using intact soil monolith lysimeters, beneath two different pasture or cropping systems: (i) perennial ryegrass/white clover pasture, and (ii) bare fallow after simulated grazing of fodder beet (beet fallow). Two different cow urine treatments (700 kg N ha^{-1}) were applied to each of the crop treatments in June 2017, using freshly collected urine from herds on a diet of either pasture or fodder beet (beet) (supplemented with lucerne silage). Winter plant growth was found to have a large effect on N leaching losses. There were high N leaching losses beneath the beet fallow (335-345 kg N ha^{-1}) compared to the significantly lower leaching loss beneath the pasture (55-121 kg N ha^{-1}). Urine treatment had a significant effect on N losses/transformations beneath the pasture. The pasture with beet urine treatment had 36% lower average ammonia oxidising bacteria (AOB) population abundance, 31% lower soil NO_3^--N concentration and subsequently 64% lower NO_3^--N leaching losses than the pasture with pasture urine treatment. The lower AOB population and lower average soil NO_3^--N suggest a biological nitrification inhibitor effect was present in the beet urine. This study shows the potential of winter plant growth and manipulation of cattle diet in reducing farm N losses.

Introduction

Temperate grazed pastoral systems are relatively inefficient at converting N entering the system into animal products, with a conversion rate of only about 15% to 35%. The large amounts of surplus N ingested by ruminants is deposited onto agricultural soils at high nitrogen loading rates (700–1000 kg N ha^{-1}), via urine patches (Selbie *et al*. 2015). This high rate of N deposited in urine patches often exceeds plant requirements, especially in winter months when plant growth and N uptake is limited. This surplus N that can be lost to the wider environment through nitrate (NO_3^-) leaching and gaseous N emissions. When drainage occurs NO_3^- can leach through the soil profile and enter surface and ground water, causing surface water eutrophication. High NO_3^- concentrations (i.e.

>11.3 ppm NO_3^--N) in drinking water also present a human health risk. There is also a significant economic cost as lost N must also be replaced through costly fertilisers.

Nitrous oxide (N_2O) emissions from agricultural soils are also of great importance, because N_2O contributes to climate change and the depletion of the ozone layer. The long term global warming potential of N_2O is 265 times that of carbon dioxide (CO_2) (Pachauri *et al*. 2014). It has also been shown that N_2O has become, and will remain, the dominant ozone-depleting substance emitted in the 21st century (Ravishankara *et al*. 2009). It is therefore necessary to improve understanding of the factors that affect these N losses and develop potential strategies to reduce them.

In temperate grazed pastoral systems cattle graze outside year round, on pasture or fodder crops, depositing N (at high rates) onto agricultural soil via urine; this leads to significant N losses. Therefore, manipulating cattle diet has been suggested as a method of reducing N losses because this can reduce urinary-N concentration (Dalley *et al*. 2017). Feeding high carbohydrate and low protein crops, such as fodder beet (beet), has therefore been suggested as a potential method to reduce urine-N concentration and subsequently reducing N losses (Lee *et al*. 2014; Dalley *et al*. 2017). Research has also shown that altering cattle diet can affect urine-carbon (C) losses (Hales *et al*. 2013). In addition, the application of C to urine patches has been used to increase the rate of N immobilisation and subsequently to reduce N leaching (Malcolm *et al*. 2019; Talbot *et al*. 2019). Therefore, altering cattle diet to increase urine-C concentration could increase immobilisation of N in urine patches; leading to a reduction in N losses. However, there is currently little published research that describes the effects of contrasting cattle diets on cattle urine and subsequent N leaching losses. More research is needed to investigate the potential of manipulating cattle diets, to alter urine-C concentration and composition, as a tool to reduce N losses.

Research has shown, in temperate climates, higher winter plant growth can decrease drainage volume and increase N uptake from the root zone, resulting in lower N leaching losses (Malcolm *et al*. 2014; Carey *et al*. 2017; Maxwell *et al*. 2018; Welten *et al*. 2019). Catch crops and winter active plants have therefore been suggested as possible methods to reduce N losses from farms. However, there is little research on the effect of beet fallow soil compared to pasture soil on key soil processes.

This study therefore will determine the effect of manipulating cattle diet and the effect of winter plant growth on N losses (NO_3^- leaching and N_2O emissions) from urine-treated soil and the effect on the key soil processes of immobilisation and nitrification (including ammonia oxidising bacteria (AOB) abundance). Our hypothesis was that increased winter plant growth will result in a higher plant uptake of soil mineral N and lower leachate drainage volume, and that higher urine-C concentrations will result in immobilisation of N; both subsequently leading to lower N losses.

Materials and methods

Lysimeter and soil block collection and installation

The experiment was conducted at Lincoln University's Ashley Dene Research and Development Station (ADRDS), near Lincoln, Canterbury (43°38'51.2"S 172°20'45.5"E; 17 m above sea level). For this experiment, 20 undisturbed soil monolith lysimeters, 500 mm in diameter and 700 mm deep, were used. Ten lysimeters were collected from a perennial ryegrass (*Lolium perenne*) cv. 'Prospect'/white clover (*Trifolium repens*) cv. 'Legacy' pasture (pasture) paddock (43°38'37.7"S 172°20'38.8"E) and 10 were collected from fodder beet (*Beta vulgaris*) cv. 'Jamon'(beet fallow) paddock (43°38'38.1"S 172°20'42.0"E). The lysimeters were collected in the summer of 2016/2017 following well-established protocols and procedures (Cameron *et al*. 1992). Between their collection and installation, the plants on the lysimeters were maintained to ensure they replicated typical paddock conditions. This was done through harvesting the pasture with electric hand shears to simulate grazing, and irrigating of the lysimeters to simulate farm irrigation practices. Each lysimeter also had a corresponding soil block, 500 mm in diameter and 200 mm deep to allow periodic removal of soil samples (Malcolm *et al*. 2019; Talbot *et al*. 2019). These soil blocks were collected in June 2017 from the same location as their corresponding lysimeters. The lysimeters and soil blocks were installed into a lysimeter trench facility at the ADRDS in June 2017. Once the lysimeters were installed, the area around them was back filled with sand at the lower depths and topsoil around the top layer. The soil blocks sat on a bed of sand and were surrounded with top soil. The top soil was levelled to the same height as the soil in the lysimeters/soil blocks.

The soil in the lysimeters and soil blocks was a Lismore/Balmoral stony silt loam (Pallic Firm Brown soil). This soil is very stony, shallow, free draining and typical of soils used for dairying in the Canterbury region of New Zealand. Soil samples were collected from 0-75 mm depth and sent for a basic soil analysis at Analytical Research Laboratories (Ravensdown, New Zealand) (Table 12).

Superphosphate fertiliser (11 g lysimeter^{-1} equivalent to 555 kg ha^{-1}) and hydrated lime (20 g lysimeter^{-1} equivalent to 1 t ha^{-1}) was applied to each pasture lysimeter and soil block on 30 June 2017. Fertiliser was applied because the Olsen P value (15) and the pH (6) of the pasture soil (0-75 mm depth) test were lower than typical dairy farm fertility levels. The fertiliser was sprinkled evenly over the lysimeters/soil blocks and then washed in to the soil by applying 4 L of water per lysimeter/soil block. A second application of hydrated lime (20 g lysimeter^{-1} equivalent to 1 t ha^{-1}) was applied to the pasture lysimeters/soil blocks on 18 September 2017. This lime was applied evenly over the lysimeters/soil blocks. The lime was applied during a rainfall event to ensure it was washed into the soil profile.

Table 12. Results of the basic soil analysis performed at the two lysimeter collection sites, at 0-75 mm depth. Pasture site (43°38'37.7"S 172°20'38.8"E), and beet fallow site (43°38'38.1"S 172°20'42.0"E). Cation exchange capacity (CEC).

	Pasture	Beet fallow
pH	6.0	6.8
Olsen P (µg g^{-1})	15	28
CEC ($mmol_c$ kg^{-1})	0.12	0.13
Exc. Ca ($mmol_c$ kg^{-1})	0.066	0.116
Exc. Mg ($mmol_c$ kg^{-1})	0.01	0.007
Exc. K ($mmol_c$ kg^{-1})	0.006	0.003
Exc. Na ($mmol_c$ kg^{-1})	0.002	0.003

Experimental design

The experiment consisted of four treatments, each with five replicates, giving a urine/crop 2 x 2 factorial design (Table 13). All lysimeters/soil blocks received urine at a rate equivalent to 700 kg N ha^{-1}. The treatments were arranged in the trench facility at ADRDS using a randomised complete block design.

Table 13. Treatments applied to the lysimeters and soil blocks.

Treatment number	Crop	Urine type	Urine rate (kg N ha^{-1})	Replicates
1	Beet fallow	Beet	700	5
2	Beet fallow	Pasture	700	5
3	Pasture	Beet	700	5
4	Pasture	Pasture	700	5

Treatments

Simulated grazing

On 30 June 2017, the pasture lysimeters/soil blocks were trimmed to a residual mass equivalent of 1500 kg DM ha^{-1} using electric shears. On the 3 July 2017, the fodder beet (beet) plants were removed from the beet fallow lysimeters/soil blocks by hand, to simulate winter grazing.

Simulated trampling of the lysimeters/soil blocks was performed on 3 July 2017. The soil in all lysimeters/soil blocks was trampled using cow hoof simulation equipment designed to provide

approximately 200 kPa that is similar to the pressure exerted by an adult cow hoof. Each pasture lysimeter/soil block received six 'stomps' from the cow hoof. The beet fallow lysimeter/soil block received 45 'stomps' from the cow hoof because beet is grazed at a higher stocking intensity (1000-1300 cows ha^{-1}) than that for pasture.

Urine collection and application

On 3 July 2017, urine was collected from two different herds of Friesian/Jersey cross cows at ADRDS. The two herds had different diets; one diet consisting mainly of pasture and the other of beet. The pasture urine was collected from 100 lactating cows, with a diet consisting of pasture (11 kg DM cow^{-1} day^{-1}) and pasture baleage (4.5 kg cow^{-1} DM day^{-1}). The beet urine was collected from 60 non-lactating cows, with a diet consisting of beet (7 kg DM cow^{-1} day^{-1}) and lucerne silage (4.5 kg DM cow^{-1} day^{-1}). Samples of the urine (50 mL) were taken and analysed for C and N concentration, using an Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany). The urine was stored overnight, in clean plastic barrels in a cool dark room at the ADRDS. On 4 July 2017, after receiving the results from the urine N concentration analyses, the pasture urine was standardised to a concentration of 6.9 g N L^{-1} using 328.4 g of urea-N in 125 L of urine, the beet urine was standardised to 6.9 g N L^{-1} using 142.7 g of urea-N in 42.5 L of urine (Table 14); of this urea-N added 89.3 g and 30.4 g was ^{15}N enriched (97.6 atom%), for pasture urine and beet urine, respectively (Table 14). This urea enriched the urine to 10 atom% ^{15}N. Each lysimeter/soil block had 2 L of the urine applied on 4 July 2017, at a rate equivalent to 700 kg N ha^{-1} using a rose head watering can, allowing an even spread of urine over the lysimeter/soil block.

Table 14. Urine nitrogen and carbon concentrations, urea-N addition and ^{15}N enrichment values.

	Beet urine	Pasture urine
Original concentrations		
Nitrogen (N) concentration (g N L^{-1})	3.7	4.6
Carbon (C) concentration (g C L^{-1})	8.6	7.2
Standardisation of N and ^{15}N enrichment		
Enrichment ^{15}N%	10	10
Mass ^{15}N enriched urea-N used (g)	30.4	89.3
Mass of standard urea-N added (g)	112.3	239.1
Final concentrations after urea addition		
Final urine N concentration (g N L^{-1})	6.9	6.9
N application rate (kg N ha^{-1})	702.8	702.8
Calculated final C concentration (g C L^{-1})	10.0	8.3
C application rate (kg C ha^{-1})	1018.6	845.4

Fodder beet cultivation, sowing and feriliser application

The beet fallow lysimeters were cultivated using hand held tools, on 24 October 2017, to simulate farm cultivation. Three beet seeds, cultivar 'Jamon', were planted into the lysimeters on 25 October 2017. The beet plants were thinned down to two plants on 23 November 2017. A fertiliser mix (0:6:15:13) was applied to the beet fallow lysimeters (12 g $lysimeter^{-1}$ equivalent to 600 kg ha^{-1}) on 7 December 2017.

Irrigation

A total of 470 mm of irrigation was applied to each lysimeter/soil block between 4 October 2017 and 3 April 2018. Irrigation was applied using a small scale pivot irrigation system at a rate of 5 to 10 mm per application. The irrigation scheduling simulated on-farm practices to replace evaporative losses.

Measurement methods

Leachate was collected from each lysimeter after each drainage event through plastic tubing attached to an outlet nozzle at the bottom of the lysimeters, leading in to 10 L collection containers. Leachate volumes were measured and 50 mL sub-samples were taken for chemical analysis. Samples were kept

frozen at -20 °C until they were analysed for ammonium (NH_4^+) and NO_3^- using a FOSS FIAstar 5000 twin channel analyser with SoFIA software version 2.00. The concentrations of ^{15}N tracer in four leachate batches was measured using a continuous flow isotope ratio mass spectrometer (IRMS) (Secron Ltd., Crewe, CW1 6JT, UK); allowing the loss of applied ^{15}N tracer in the leachate to be calculated. Samples were prepared using the diffusion procedure described by Brooks *et al*. (1989).

Gas samples were collected using a closed chamber method similar to that described in Hutchinson and Mosier (1981). The gas chambers were constructed from a metal cylinder insulated with 25 mm thick polystyrene foam to avoid heating of the atmosphere in the chamber during sampling. The chamber was placed inside a small water trough which was mounted around the top of each lysimeter casing. The chamber was placed there for 40 minutes during which time three 20 mL gas samples were collected, 20 minutes apart. The gas samples were collected using a syringe through a rubber septum on top of the gas chamber. Samples were collected twice weekly for the first three months after urine application and then once weekly for the fourth month after urine application. The N_2O concentration of the gas samples was measured using a gas chromatograph (Model 8610C, SRI Instruments, California, USA) linked to a Gilson GX-271 autosampler (Gilson Inc, MI, USA). Once weekly throughout the measurement period, additional samples were collected for ^{15}N tracer analysis. Following the regular sampling (described above), chambers remained on the lysimeters for a total of 3 hours, after which a gas sample (40 mL) was collected. The ^{15}N tracer enrichment of N_2O emissions from three batches were analysed, using IRMS (Secron Ltd., Crewe, CW1 6JT, UK). Gas samples were analysed using a TGII trace gas system, equipped with cryo-trapping and focusing, to isolate the fraction of interest.

The pasture lysimeters were harvested when plant development had reached the 2-3 leaf stage and yields were approximately 3000 kg DM ha^{-1}. The herbage was cut to a residual height of approximately 50 mm (1500 kg DM ha^{-1}). The beet was harvested once on 3 July 2018, at the conclusion of the experiment. The beet were removed from the ground by hand, washed to remove soil and were separated into leaves and bulb. All herbage samples were collected into paper bags. Dry matter production was determined gravimetrically after drying at 70°C for 72 hours (beet bulb was dried for 144 hours). The dry herbage samples were then ground using a Retsch Ultra Centrifugal Mill ZM 200, with a 1 mm sieve and running at a speed of 18,000 rpm. The samples were stored in sealed 70 mL containers at room temperature in the dark, until analysis for their total N content using an Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany). The ^{15}N enrichments for the herbage were determined using IRMS. Analyses for ^{15}N tracer concentrations were performed on five of the nine pasture harvests and on the one beet harvest.

Soil samples were taken from the soil blocks to elucidate the mechanisms involved in the transformation of soil mineral N. Samples were taken using a soil corer (100 mm depth, 75 mm diameter) on days 0, 1, 7, 14, 28, 56 and 112 after urine application. Samples were collected on day 0, prior to urine application, allowing changes to be compared to day 0 as a baseline. Holes remaining after core removal were immediately back-filled with a soil-sand mix and identified using plastic markers to prevent subsequent samplings occurring from that same position. The samples were stored at -80°C until analysis. Soil mineral N (NO_3^- and NH_4^+) concentration was determined on potassium chloride (KCl) extracts on a Lachat QuikChem 8500 Series 2 Flow Injection Analysis System (Lachat Instruments, Loveland, CO, USA), as described in Talbot *et al*. (2019).

In agricultural soil, nitrification is largely driven by soil ammonia oxidising bacteria (AOB) and ammonia oxidising archaea play a lesser role (Di *et al*. 2009b). Therefore, DNA extraction and soil AOB ammonia monooxygenase (amoA) gene abundance was measured using quantitative polymerase chain reaction (qPCR), following methodologies adapted from (He *et al*. 2007) and described in Talbot *et al*. (2019). In brief, DNA was extracted from the soil using a NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany) as per the manufacturer's instructions. AOB amoA gene abundance was measured using real time qPCR on a Rotor-Gene™ 6000 (Corbett Research, Australia). Data were then analysed using the Rotor-Gene™ series software 1.7.

Recoveries of ^{15}N (%) in herbage, leachate and N_2O emissions were calculated using the equation from Cabrera and Kissel (1989). A value of 0.3663% was used as the B value (atom % ^{15}N natural abundance enrichment). Maximum (0.3700%) and minimum (0.3637%) B values from literature were also used, however, this had no significant effect on the dataset (Dürr & Mary 1998; Hansen & Vinther 2001; Stevenson *et al*. 2010). Interpolation was used to estimate the ^{15}N enrichment for those sampling occasions when ^{15}N analyses were not performed. For N_2O emissions, the ^{15}N recovery at each sampling date was integrated to estimate the total ^{15}N recovery during the sampling period.

Statistical analysis

The data sets were subject to analysis of variance (ANOVA) using Genstat (18th edition, VSN International Ltd.). For each ANOVA, orthogonal contrasts (2 x 2 factorial) were used to determine the significance of the main effect of crop type (pasture vs beet fallow) and of urine type (pasture urine vs beet urine) and their interaction (crop x urine type). The NO_3^--N leaching, NH_4^+-N leaching, total mineral N leaching, and ^{15}N leachate recovery data sets were log transformed to ensure homogeneity of residual errors.

Results

Climate conditions and water inputs

Over the experimental period (4 July 2017 - 3 July 2018), the average daily air temperature ranged from a low of 0.8 °C in June 2018 to a high of 23.7 °C in December 2017 (Figure 27a). Daily average soil temperature (100 mm depth) ranged from a low of 3.4°C in July 2017 to a high of 27.9°C in December 2017 (Figure 27a). Water inputs over the 12 month experimental period totalled 1363 mm, comprising 877 mm of rainfall, 470 mm of irrigation and 16 mm of water input from treatments (Figure 27b). The 12 month period had 42% higher rainfall than the long-term average (618 mm), due to a very wet winter in 2017.

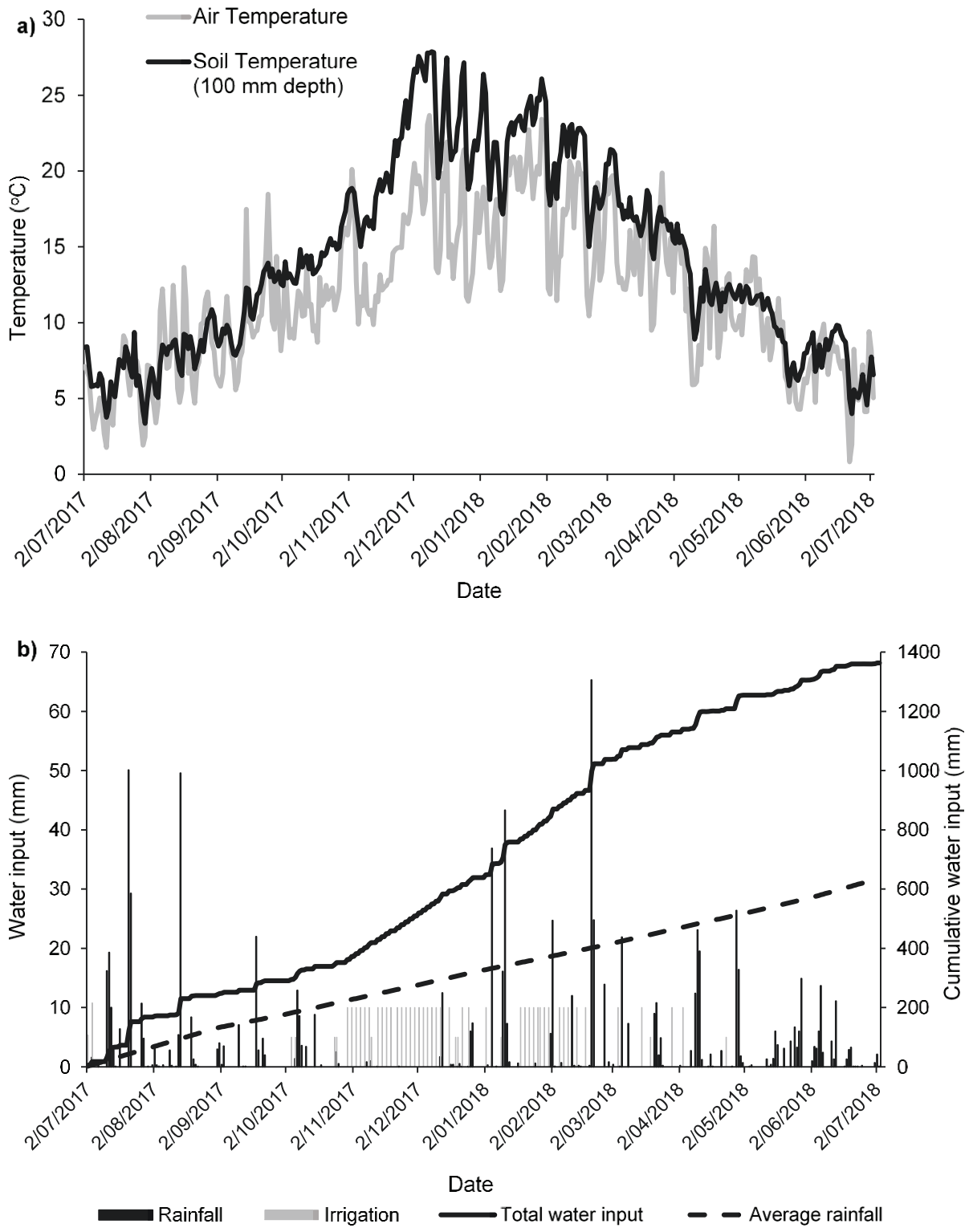

Figure 27. a) Mean daily air temperature and soil temperature (100 mm depth), and b) daily rainfall, irrigation water inputs and cumulative water input over the experimental period (4 July 2017 - 3 July 2018), and long-term average rainfall data (1971-2000, Lincoln weather station 4881).

Leaching losses

Nitrate concentration curve

The NO_3^- leaching breakthrough curves show peak concentrations at 226.4, 240.9, 28.1 and 55.9 mg NO_3^--N L^{-1} under the beet fallow with beet urine, beet fallow with pasture urine, pasture with beet urine, and pasture with pasture urine treatments, respectively (Figure 28). These NO_3^--N concentration peaks occurred between 230-290 mm of cumulative drainage.

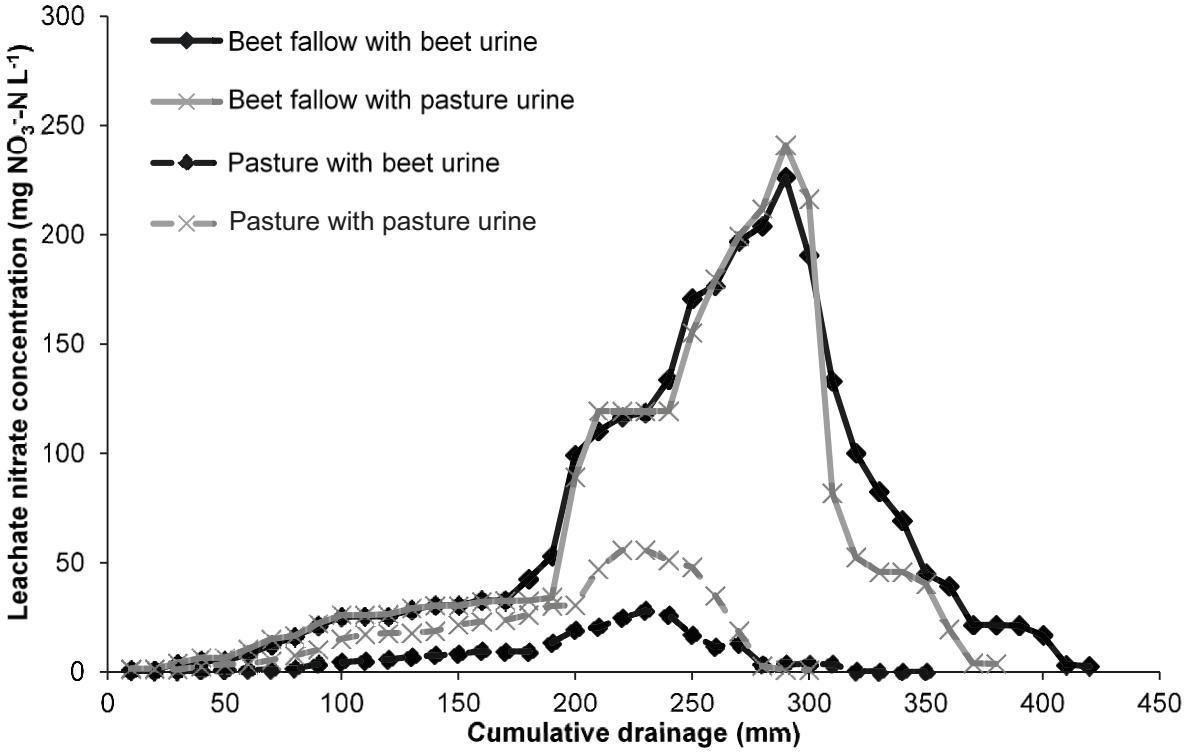

Figure 28. Mean nitrate concentration in leachate plotted against cumulative drainage (mm) over the sampling period (4 July 2017- 17 May 2018), under different treatments.

Total leaching losses

The effects of each treatment on total log transformed NO_3^--N, NH_4^+-N and mineral N leached over the sampling period (4 July 2017 – 17 May 2018) and the total amount of annual drainage (4 July 2017 – 3 July 2018) are shown in Table 15.

The pasture leached less NO_3^--N than the beet fallow ($P < 0.001$) and the beet urine treatment leached significantly ($P = 0.047$) less NO_3^--N than the pasture urine treatment (Table 15). There was also a significant ($P = 0.023$) interaction between crop and urine, with the pasture with beet urine treatment leaching 64% less NO_3^--N than the pasture with pasture urine treatment, while no significant difference was observed between the beet fallow with beet urine and beet fallow with pasture urine treatments (Table 15).

Table 15. The effect each treatment had on total log nitrate, log ammonium and log mineral N leached over the sampling period (4 July 2017- 17 May 2018) and the total amount of annual drainage (4 July 2017- 3 July 2018). Values with a subscript letter in common are not significantly different at the 5% level. The first three contrasts are main effect (m.e.) and interaction contrasts for a 2 x 2 factorial, with factors crop type and urine type.

		Log_{10} means (Brackets contain back transformed means)						
Crop	**Urine type**	**Nitrate leached (kg NO_3^--N ha^{-1})**		**Ammonium leached (kg NH_4^+-N ha^{-1})**		**Mineral N leached (kg N ha^{-1})**		**Drainage volume (mm)**
Beet fallow	**Beet**	2.427	(**267**)^a	1.812	(**65**)^a	2.525	(**335**)^a	543^a
	Pasture	2.390	(**246**)^a	1.994	(**99**)^a	2.538	(**345**)^a	522^a
Pasture	**Beet**	1.311	(**20**)^c	1.435	(**27**)^b	1.743	(**55**)^c	457^a
	Pasture	1.754	(**57**)^b	1.777	(**60**)^a	2.081	(**121**)^b	480^a
LSD (5%) (*n*=5)		0.283		0.326		0.241		88
P values for contrasts	Crop (Beet fallow vs pasture) m.e.	<0.001		0.017		<0.001		0.043
	Urine (Beet vs pasture) m.e.	0.047		0.030		0.045		0.967
	Interaction: Crop x Urine	0.023		0.457		0.059		0.450

There were significant crop ($P = 0.017$) and urine ($P = 0.030$) effects on NH_4^+-N leaching. The beet fallow had greater NH_4^+-N leaching loss than that for the pasture and the pasture urine treatment had greater NH_4^+-N leaching loss than that for the beet urine treatment.

There was a significant crop ($P < 0.001$) and urine ($P = 0.045$) effect on mineral N leaching. The pasture overall had lower total mineral N leached than that for the beet fallow, and the pasture urine overall had greater mineral N leaching loss than that for the beet urine. The pasture with beet urine treatment leached significantly less mineral N than the pasture with pasture urine treatment (by 55%), however, there was no difference between the beet fallow treatments (Table 15).

Crop type had a significant effect on total annual drainage (P = 0.043) and urine type had no effect on drainage (P = 0.967). Overall the pasture had 12% lower drainage than the beet fallow.

Nitrous oxide emissions

There were no significant main effects of urine type (P = 0.551) or crop type (P = 0.281) on N_2O-N emissions over the sampling period (Figure 29).

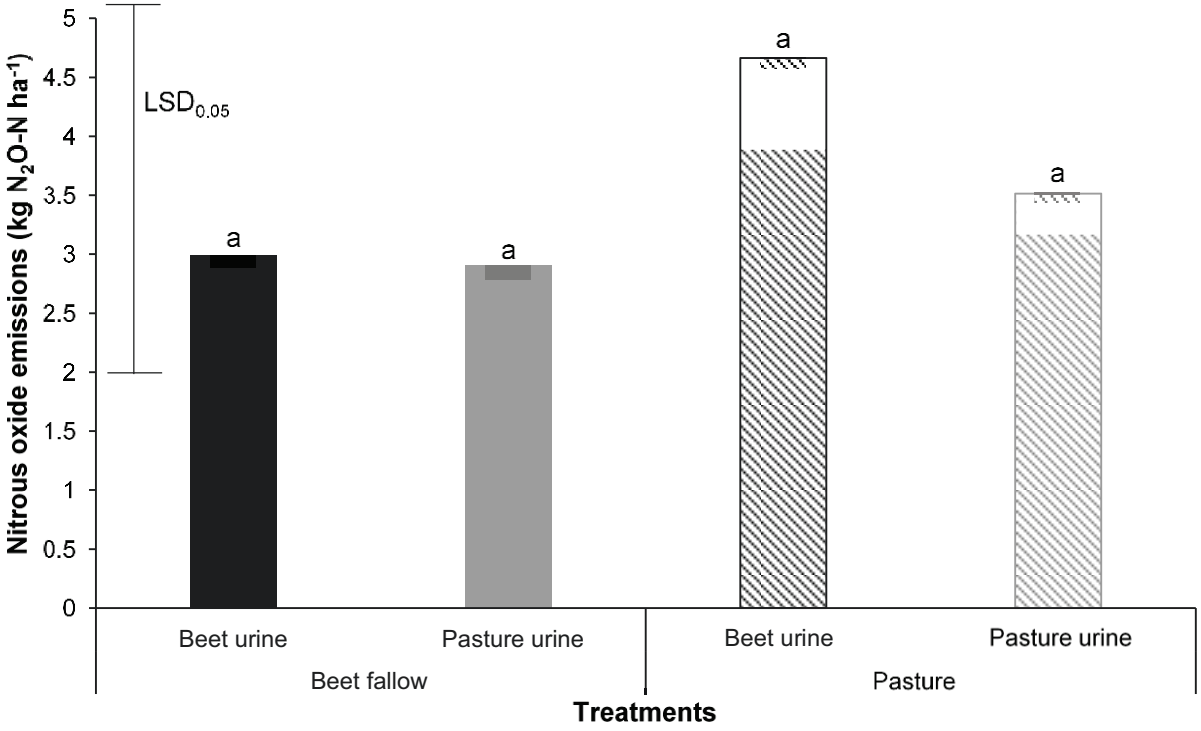

Figure 29. Treatments effects on nitrous oxide emissions over the sampling period (4 July 2017 - 31 October 2017). Least significant difference (LSD) is at the 5% level (*n* = 5). LSD = 3.1. Bars with a letter in common are not significantly different at the 5% level.

Herbage yield and nitrogen uptake

Yield

There was no significant crop (P = 0.696) or urine (P = 0.130) effects on annual yield throughout the experimental period (Figure 30a). However, the yield was significantly higher for the pasture with beet urine treatment (17,321 kg DM ha^{-1}) than that for the pasture with pasture urine treatment (15,096 kg DM ha^{-1}) (Figure 30a).

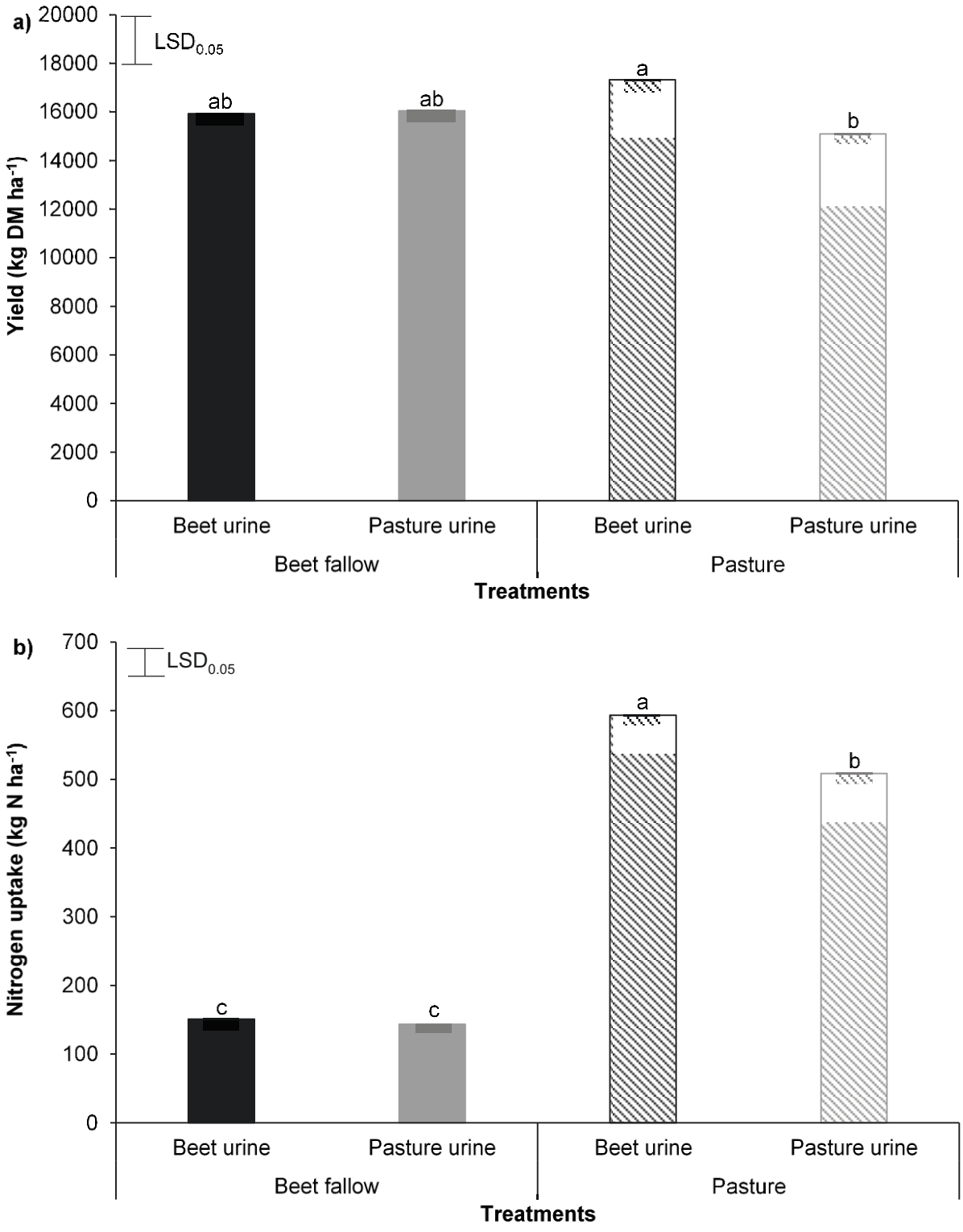

Figure 30. Treatment effects on a) annual yield and b) annual nitrogen uptake. Least significant difference (LSD) is at the 5% level (*n* = 5). a) LSD = 1951.7 b) LSD = 42.3 Bars with a letter in common are not significantly different at the 5% level.

Plant nitrogen uptake

There was a significant crop ($P < 0.001$) and urine ($P = 0.007$) effect on annual plant N uptake over the experimental period (Figure 30b). The pasture had significantly higher annual plant N uptake than that for the beet fallow and the beet urine treatment had significantly greater N uptake than that for the pasture urine treatment. There was also a significant interaction between crop and urine ($P = 0.013$), with the pasture with beet urine treatment (594.5 kg N ha^{-1}) resulting in significantly higher annual plant N uptake than that of the pasture with pasture urine treatment (508.7 kg N ha^{-1}), however, there was no difference between the beet fallow treatments.

Soils

Mineral N

Mineral N was highest in all treatments immediately following urine application (Figure 31). Thereafter, a continual decrease in soil mineral N was observed, under all treatments. Averaging over the period from day 1 to 112 after urine application using the trapezoid rule to calculate the area under the curve (AUC) and dividing by 112, the average soil mineral N, NO_3^--N and NH_4^+-N could be calculated for each treatment. Crop type was shown to have a highly significant ($P < 0.001$) main treatment effect on average soil mineral N. The beet fallow had significantly higher average soil mineral N than that for the pasture. The urine treatment had no significant effect on total average soil mineral N ($P = 0.997$).

However, urine type had a significant effect on the soil mineral N compostion beneath the pasture, with a significant difference in average soil NO_3^--N between the two urine types ($P = 0.026$). The average soil NO_3^--N was significantly higher for the pasture with pasture urine treatment (59.3 kg NO_3^--N ha^{-1}) than that for the pasture with beet urine treatment (40.9 kg NO_3^--N ha^{-1}) (Figure 31). The average soil NH_4^+-N was slightly lower for pasture with pasture urine treatment (20.8 kg NH_4^+-N ha^{-1}) than that for the pasture with beet urine treatment (30.7 kg NH_4^+-N ha^{-1}), however, this difference was not significant ($P = 0.175$).

AOB gene abundance

The average AOB *amoA* gene abundance was significantly ($P = 0.005$) lower for the pasture with beet urine treatment (2.2E+06 *amoA* gene number g^{-1} dry soil) than that for the pasture with pasture urine treatment (3.4E+06 *amoA* gene number g^{-1} dry soil) (Figure 32). The pasture was shown to have significantly lower average AOB *amoA* gene abundance than that for the beet fallow ($P < 0.001$) (Appendix F).

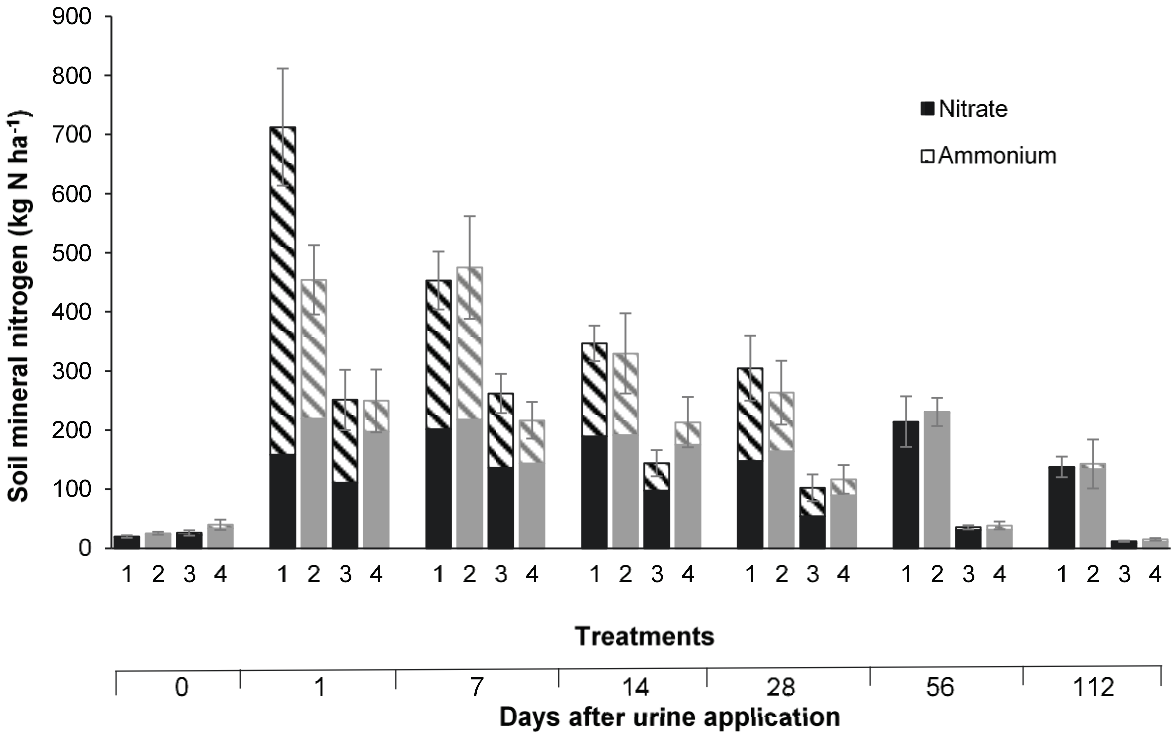

Figure 31. Treatment effects on the soil mineral nitrogen (NO_3^--N + NH_4^+-N) over the sampling period (4 July 2017- 24 October 2017). Day 0 is prior to urine application. Soil samples were taken from 0-100 mm depth. Treatments: 1 = Beet fallow with beet urine; 2 = Beet fallow with pasture urine; 3 = Pasture with beet urine; 4= Pasture with pasture urine. Error bars are standard error of the mean for total mineral nitrogen (*n* = 5).

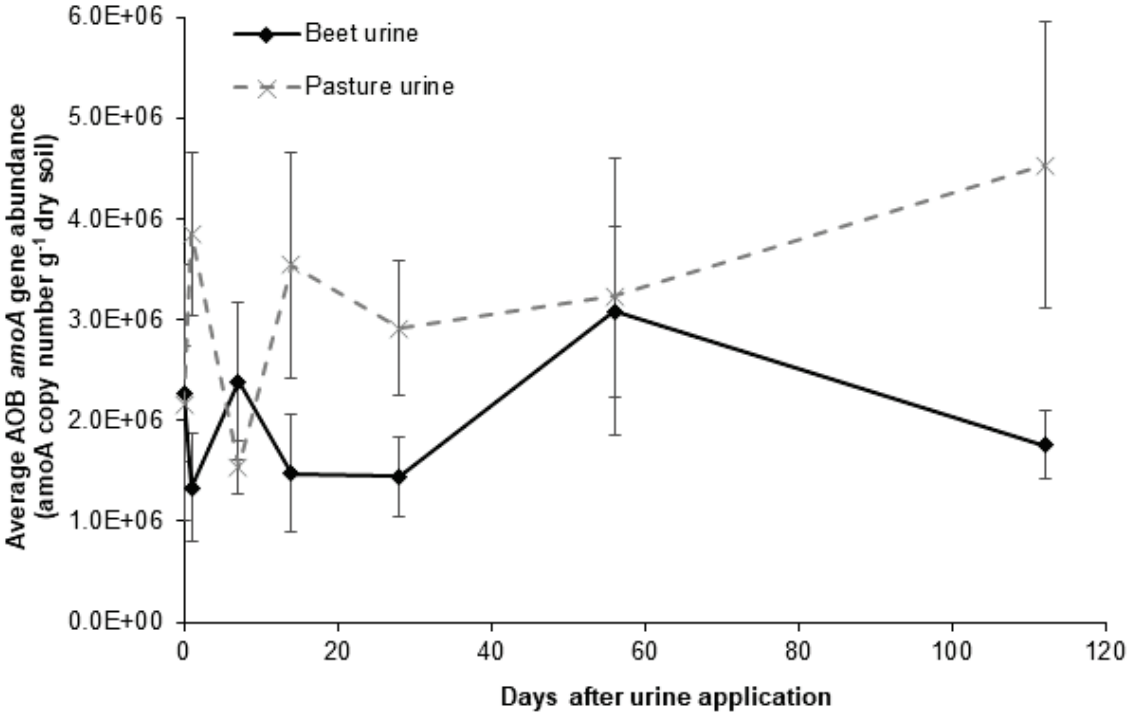

Figure 32. The effect of urine type on soil ammonia oxidising bacteria (AOB) *amoA* gene copies over the first 112 days after urine application, beneath pasture. Day 0 is prior to urine application. Samples were taken from 0-100 mm depth in the soil profile. Error bars are standard error of the mean (*n* = 5).

^{15}N recovery

The effect each treatment had on ^{15}N recovery % of the ^{15}N applied among the herbage, log leachate and N_2O emissions is shown in Table 16. There was a highly significant ($P < 0.001$) crop effect on ^{15}N herbage recovery, with the pasture having significantly higher ^{15}N herbage recovery than that for the beet fallow. Beneath the beet fallow, urine type had no significant effect on ^{15}N herbage recovery. However, the ^{15}N herbage recovery was significantly higher for the pasture with beet urine treatment than the pasture with pasture urine treatment.

There was a significant crop effect ($P < 0.001$) and a near significant urine effect ($P = 0.059$) on ^{15}N leachate recovery. The ^{15}N leachate recovery was significantly greater beneath the beet fallow than that for the pasture (Table 16). Beneath the beet fallow, urine type had no significant effect on ^{15}N leachate recovery. However, the ^{15}N leachate recovery was significantly lower for the pasture with beet urine treatment (6.3%) than that for the pasture with pasture urine treatment (13.4%) (Table 16). There was no significant crop effect (P =0.416) or urine effect ($P = 0.535$) on ^{15}N N_2O emission recovery.

Table 16. Recovery (%) of the ^{15}N (applied with the urine) in the herbage, leachate, and nitrous oxide fractions. Values with a subscript letter in common are not significantly different at the 5% level. The first three contrasts are main effect (m.e.) and interaction contrasts for a 2 x 2 factorial, with factors crop type and urine type. Baseline ^{15}N enrichment was assumed at 0.3663%.

Crop	Urine type	Herbage	Log Leachate (Back transformed data)		N_2O emissions
Beet fallow	**Beet**	**13.1^{c}**	1.572	(**37.3**)^{a}	**0.275^{a}**
	Pasture	**13.2^{c}**	1.579	(**37.9**)^{a}	**0.261^{a}**
Pasture	**Beet**	**43.5^{a}**	0.798	(**6.3**)^{c}	**0.394^{a}**
	Pasture	**36.5^{b}**	1.127	(**13.4**)^{b}	**0.294^{a}**
LSD (5%) (*n*=5)		6.1	0.249		0.275
P values for contrasts	Crop (Beet fallow vs pasture) m.e.	<0.001	<0.001		0.416
	Urine (Beet vs pasture) m.e.	0.106	0.059		0.535
	Interaction: Crop x Urine	0.102	0.070		0.640

Discussion

Crop effect

Crop type was found to have a significant effect on soil mineral N, AOB *amoA* gene abundance, plant N uptake, and N leaching values. The relatively winter active pasture was shown to have significantly lower average soil mineral N than that for the beet fallow, for the first 112 days after urine application (P <0.001). This is attributed to higher winter plant growth and subsequently higher plant N uptake from the pasture (Figure 30). The lower soil mineral N beneath the pasture may have limited the AOB *amoA* gene abundance, as the AOB are autotrophic bacteria that obtain their energy from the oxidation of NH_4^+-N (Kowalchuk & Stephen 2001). The lower amount of soil NH_4^+-N and the limitation of AOB population growth, beneath the pasture, reduced the amount of soil NO_3^--N produced, compared with values for the beet fallow (Figure 31). This subsequently led to a reduction in NH_4^+-N (P = 0.017), NO_3^--N (P <0.001) and total mineral N (P <0.001) leaching beneath the pasture, compared with values for the beet fallow (Table 15). The pasture leached 84% and 65% less mineral N than that for the beet fallow, under the beet urine and pasture urine treatments, respectively.

The values and effect of plant winter growth on N leaching losses are consistent with those in the literature for temperate climates where rainfall is not limiting plant growth. Other studies suggests that mineral N leaching losses beneath a pasture with traditional pasture urine (700 kg N ha^{-1}) applied, to be between 69-306 kg N ha^{-1} (Cameron *et al*. 2007; Carlton *et al*. 2018; Woods *et al*. 2018). This is consistent with the 121 kg N ha^{-1} shown in this study. There is very limited literature on N leaching losses under a beet fallow. One study that has measured N leaching under beet fallow is Malcolm *et al*. (2016) where N leaching losses (64-84 kg NO_3^--N ha^{-1}) were much lower under beet fallow than those for this study (246-267 kg NO_3^--N ha^{-1}). However, in Malcolm *et al*. (2016) urine applications were only 250-300 kg N ha^{-1}, significantly lower than the 700 kg N ha^{-1} applied in this study. It is therefore not unexpected that Malcolm *et al*. (2016) detected lower N leaching losses. The urine application rate of 250-300 kg N ha^{-1}, in Malcolm *et al*. (2016), is a more realistic value under beet urine patches; due to the low rates of N excretion from cows grazing beet. However, a standard rate of 700 kg N ha^{-1} was used in this study to allow investigation of urine composition effect. High levels of N leaching (172-380 kg N ha^{-1}), similar to this study, however, are shown in the literature under bare fallow kale, with urine applied (350-700 kg N ha^{-1}) (Malcolm *et al*. 2015a; Carey *et al*. 2016). The ^{15}N tracer results strengthen these findings, showing crop type had a significant effect on ^{15}N leachate and herbage recovery (Table 16).

In the literature there are multiple ^{15}N tracer studies under pasture, but none under beet fallow. The pasture ^{15}N recovery rates (herbage 36.5-43.5%, leachate 6.3-13.4% and N_2O emissions 0.3-0.4%) were relatively consistent with those in literature (herbage 21.6-43.5%, leachate 6-31.7% and N_2O emissions

0.5-1.0%) (Fraser *et al*. 1994; Clough *et al*. 1998; Di *et al*. 2002; Silva *et al*. 2005; Welten *et al*. 2013a; Selbie 2014; Woods *et al*. 2017).

This study shows that N leaching losses were particularly high in the beet fallow treatment, largely due to soils remaining in a bare fallow state for an extended period of time after beet grazing, consistent with usual practice on farm (Table 15). These results, with the supporting literature, highlight the importance of winter growth in reducing N leaching losses (Malcolm *et al*. 2014; Maxwell *et al*. 2018; Welten *et al*. 2019). The large N leaching losses under the beet fallow and the reduction in N leaching losses under winter active pasture also highlight the potential of catch crops to reduce farm N leaching losses (Carey *et al*. 2016; Carey *et al*. 2017, 2018; Malcolm *et al*. 2018). Increasing use of winter active plants on farms and selectively breeding winter active cultivars are also potential options for reducing N leaching losses from temperate grazed pastoral systems.

Urine effect

The effect urine type had on N losses/uptakes and transformations were markedly different beneath pasture and beet fallow. Urine type had no significant effects on N losses/uptakes and transformation beneath the beet fallow. This is attributed to the high N leaching loss beneath the bare fallow beet soil making the urine effect insignificant. However, beneath the pasture, urine type had a significant effect on NH_4^+-N, NO_3^--N and total mineral N leaching, annual yield and N uptake, average AOB *amoA* gene abundance, average soil NO_3^--N, and ^{15}N herbage and leachate recovery. Therefore, the discussion below focusses on the effect urine type had on nitrogen transformations beneath the pasture.

Beneath the pasture, urine type had no significant effect on average total soil mineral N, over the first 112 days after urine application (P = 0.314). This lack of a significant decrease in average soil mineral N, suggests that the different urine-C concentrations (Beet urine: 1018.6 kg C ha^{-1}. Pasture urine: 845.4 kg C ha^{-1}) made no significant effect on immobilising soil mineral N. However, urine type did have a significant effect on the composition of the soil mineral N. The average soil NO_3^--N was significantly lower for the beet urine treatment (40.9 kg NO_3^--N ha^{-1}) than that for the pasture urine treatment (59.3 kg NO_3^--N ha^{-1}) (P = 0.026). The average soil NH_4^+-N was higher for the beet urine treatment (30.7 kg NH_4^+-N ha^{-1}) than that for the pasture urine treatment (20.8 kg NH_4^+-N ha^{-1}), however, the difference was not significant (P = 0.173). The significantly lower average soil NO_3^--N and slightly higher average soil NH_4^+-N values are attributed to the significantly lower average AOB *amoA* gene abundance for the beet urine treatment compared to that for the pasture urine treatment (P = 0.005). The lower AOB population for the beet urine treatment, compared with that for the pasture urine treatment, would result in lower nitrification rates and subsequently lower average soil NO_3^--N and higher average soil NH_4^+-N. The 64% reduction in NO_3^--N leaching under the beet urine treatment, compared with that for the pasture urine treatment, was a result of the lower average soil NO_3^--N concentration. Even

though the beet urine had lower average soil NO_3^--N, there was no significant urine effect on N_2O-N emissions (Figure 29).

The beet urine treatment had significantly greater N uptake and yield than that for the pasture urine treatment (Figure 30). The beet urine treatment also had significantly higher ^{15}N herbage recovery than that for the pasture urine treatment (Table 16). The greater N uptake under the beet urine treatment is attributed to the lower N leaching losses and the higher levels of average soil NH_4^+-N, than those for the pasture urine treatment. The higher N leaching losses under the pasture urine treatment reduced the amount of urinary-N that is available for the pasture to uptake over the experimental period. The slightly higher levels of soil NH_4^+-N under the beet urine treatment, was also attributed to the higher plant N uptake as perennial ryegrass (RG) has been shown to significantly prefer to uptake NH_4^+ over NO_3^- in cooler conditions (Clarkson *et al*. 1986). This increased plant uptake in N (in particular NH_4^+-N) can be attributed to the reduction in NH_4^+-N leaching (Table 15).

The lower AOB *amoA* gene abundance and subsequently lower levels of soil NO_3^--N, suggest that the beet urine has a biological nitrification inhibitor (BNI) effect. A BNI effect in the beet urine explains the inhibited AOB population growth, the subsequently lower levels of soil NO_3^--N, and the 64% reduction in NO_3^--N under the beet urine treatment, compared with values for the pasture urine treatment. We hypothesize there could be a BNI present in the beet crop, which when eaten by cattle, enters the bloodstream and is passed through the kidneys (without losing the BNI effect), and is excreted through urine; or there could be BNI-precursors in the beet crop, which could become active BNI-compounds in the digestive tract of the cattle, which are then excreted through urine. Evidence supporting the presence of BNIs in wide variety of ecosystems and in the tissues of plants has been found (Subbarao *et al*. 2007b). However, the literature on BNIs in urine is limited (Luo *et al*. 2015b; Di & Cameron 2016; Judson *et al*. 2018; Yao *et al*. 2018). Di *et al*. (2016) and Yao *et al*. (2018) both suggested that beet urine contains compounds which affect the N transformation processes, after finding a reduction in N_2O emissions under beet urine treatment, compared to that for a kale urine treatment. Luo *et al*. (2015b) suggested that urine from sheep grazing forage rape (*Brassica napus L*.) contained plant secondary metabolites that affected N transformation processes, after finding a reduction in the N_2O emission factor under forage rape urine, compared to that for a RG urine treatment. A BNI in urine was also suggested by Judson *et al*. (2018); who found that urine from sheep fed plantain diets reduced the rate of nitrification in soil, throughout the 28 days after application, compared with urine from sheep fed RG. The results of this study along with literature, highlight the potential of manipulating urine-C composition to reduce N leaching losses, while not significantly increasing N_2O-N emissions.

The urine in this experiment was also applied at a standard concentration (6.9 mg N L^{-1}). However, if applied at actual concentrations, the beet urine treatment (3.7 mg N L^{-1}) would have had lower urinary-

N concentration than that for the pasture urine treatment (4.6 mg N L^{-1}). This would compound the reduction in N losses under the beet urine treatment, compared with that for the pasture urine treatment. For these reasons, this study shows the potential benefits of feeding cattle a diet consisting mainly of beet. The benefit of the beet urine could also be coupled with the benefit of winter growth to reduce N leaching. This could be done through feeding cattle a diet consisting of mainly beet, while grazing on a winter active crop. This would allow the beet diet to reduce urinary-N and cause a BNI effect; while a winter active crop can uptake the urinary-N at a higher rate. This strategy could potentially be an economical method to significantly reduce N leaching losses from farms, while not having a significant effect on N_2O emissions. Further research is needed to explore the potential to manipulating urine-C composition through diet manipulation, to reduce N leaching losses from urine patches.

Conclusions

Winter plant growth was shown to be extremely important for reducing N leaching losses. The large N leaching losses under the beet fallow and the reduction in N leaching losses under pasture highlights the potential of winter active plants as an opportunity to reduce farm N leaching losses from temperate grazed pastoral systems. Urine type also had a significant effect on N losses, because the results suggest the presence of a biological nitrification inhibitor in the beet urine, this provides an opportunity to manipulate cattle diet to reduce N leaching losses. This could be achieved through feeding cattle a diet consisting mainly of beet, while grazing on a winter active crop. This would allow the beet diet to reduce urinary-N concentration and cause a BNI effect, coupled with a winter active crop to take up the urinary-N at a high rate.

Further research is needed to identify the compound(s) responsible for the BNI effect observed under the beet urine treatment. Research is also needed to identify more pastures/crops that are winter active and/or produce a BNI effect, via urine application or directly in the soil.

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Chapter 5

Lysimeter experiment 2

Effect of plantain, urine-N loading rate and plantain derived urine on nitrogen losses from a shallow stony soil

5.1 Introduction

In New Zealand dairy farming systems, cattle urine patches are the main source of N losses. This is due to the high N rates deposited in urine patches (approximately 700 kg N ha^{-1}) (Selbie *et al*. 2015). The N in urine patches can undergo nitrification and be lost through NO_3^- leaching or undergo denitrification and be lost as N_2O or N_2 emissions. These losses have negative environmental impacts; NO_3^- can cause eutrophication in freshwater and N_2O is a potent greenhouse gas and ozone depleting substance. There is also an economic impact for farmers who must replace the lost N through increased application of fertilisers. It is for these reasons, that N losses from farmland must be reduced.

One potential strategy for reducing N loss from agricultural systems is the inclusion of plantain (*Plantago lanceolate* L.) in the pasture species mix. Plantain has been proposed as a means of reducing nitrate leaching due to its effect in reducing urinary-N loading rates and its ability to manipulate the N cycle (Dietz *et al*. 2013; Box *et al*. 2017; Mangwe *et al*. 2019). Recent research suggests plantain manipulates the N cycle through the production of biological nitrification inhibitors (BNI), having diuretic properties and lowering N concentration in urine (Box *et al*. 2017; Cheng *et al*. 2017a; Carlton *et al*. 2018). It has been suggested that plantain has the ability to release nitrification inhibiting root exudates, which cause BNI (Rauber *et al*. 2008; Dietz *et al*. 2013; Massaccesi *et al*. 2015; Carlton *et al*. 2018). Biological nitrification inhibitors reduce nitrification rates, which can deliver a reduction in N leaching losses. Diets containing plantain have also been shown to reduce urinary N concentration (Totty *et al*. 2013; Edwards *et al*. 2015; Box *et al*. 2016; Judson & Edwards 2016; Box *et al*. 2017; Bryant *et al*. 2017; Minneé *et al*. 2017). This reduces the N load of urine patches, thus reducing N leaching. However, there are some studies which found no difference in urine N concentrations between pastures with and without plantain (Cheng *et al*. 2017b; Bryant *et al*. 2018). Some research also indicates plantain is a diuretic for sheep (O'Connell *et al*. 2016) and cattle (Cheng *et al*. 2017a).

For these reasons the current literature suggests incorporating plantain into traditional pastures is a potential tool for reducing both N leaching (Beukes *et al*. 2011; Woodward *et al*. 2013; Beukes *et al*. 2014; Romera *et al*. 2017) and N_2O emissions (Di *et al*. 2016; Gardiner *et al*. 2016) from farms. However, the literature is still limited, with much of the research focused on diverse pastures which

also include chicory, lucerne and Italian ryegrass. There are currently no N leaching or N_2O emission values for plantain incorporated pasture under shallow stony soils. These soils are typical of 70% of Canterbury's area and are therefore of great importance to NZ agriculture (Carrick *et al*. 2013). Therefore the objectives of this study were to: (i) quantify the effect of plantain in pasture and (ii) the effect of urine from cows grazing on plantain had on N leaching losses, N_2O emissions and the AOB *amoA* gene abundance in soil.

5.2 Methods

5.2.1 Lysimeter/soil block collection and installation

In the summer of 2017/18, 40 lysimeters (500 mm in diameter and 700 mm deep) were collected from ADRDS, using the same methodology as described in Section 3.2. Twenty lysimeters were collected from a perennial ryegrass (*Lolium perenne* L.) cv. 'Prospect' and white clover (*Trifolium repens* L.) cv. 'Legacy' (PRG/WC) paddock (43°38'37.7"S 172°20'38.8"E) and twenty lysimeters were collected from a paddock (43°38'42.9"S 172°20'51.0"E) containing plantain (*Plantago lanceolate* L.) cv. 'Tonic' and white clover (T*rifolium repens* L.) cv. 'Legacy' (Appendix E). Each lysimeter had a corresponding soil block (500 mm in diameter and 200 mm deep) collected from the same location as each corresponding lysimeter, using the methodology described in Section 3.2. These lysimeters/soil blocks were maintained, with regular herbage removal and irrigation, under typical farm conditions until the start of the experiment. The lysimeters/soil blocks were installed into a trench facility at ADRDS (Figure 33), in April 2018, using methodology described in Section 3.2.

Figure 33. Lysimeter experiment 2 - lysimeters and soil blocks installed in the trench facility at ADRDS.

Soils and fertility

The soil where the lysimeters/soil blocks were collected was a Balmoral stony silt loam (Pallic Firm Brown soil) (Landcare Research 2016a). The soil profile in the plantain/white clover paddock was identical to the soil profile in the PRG/WC paddock (Appendix E). The basic fertility of the soil was

tested at both lysimeter/soil block collection sites, to ensure the soils were of similar fertility. The soil samples (0-75 mm depth) were collected 27 February 2018 and sent to Analytical Research Laboratories (Ravensdown, New Zealand) for basic soil analysis. The soil samples yielded the following results (Table 17).

Table 17. Soil test results of the paddocks where the lysimeters/soil blocks were collected.

	Perennial ryegrass/white clover (43°38'37.7"S 172°20'38.8"E)	**Plantain/white clover** (43°38'42.9"S 172°20'51.0"E)
pH	6.0	6.3
Olsen P (mg L^{-1})	16	35
Exchangeable Ca^{2+} (me 100 g^{-1})	6.3	12.5
Exchangeable Mg^{2+} (me 100 g^{-1})	1.0	0.7
Exchangeable K^{+} (me 100 g^{-1})	0.5	0.7
Exchangeable Na^{+} (me 100 g^{-1})	0.3	0.2
Sulphate sulphur (µg g^{-1})	2	1
Cation exchange capacity (me 100 g^{-1})	12	16

Fertiliser was applied to the lysimeters and soil blocks on 14 March 2018. Superphosphate (14.4 g lysimeter^{-1} equivalent to 778 kg ha^{-1}) was applied to each PRG/WC lysimeter and soil block, as the Olsen P (16) and the Sulphate Sulphur (2) values from the soil test were low. The fertiliser was sprinkled evenly over the lysimeters/soil blocks and was washed into the soil by applying 2 L of water per lysimeter or soil block. Gypsum (8.8 g lysimeter^{-1} equivalent to 475 kg ha^{-1}) was applied to each plantain/white clover lysimeter and soil block, as the sulphate sulphur (1) value from soil test was low. The fertiliser was dissolved in 2 L of water and applied using a rose head watering can.

5.2.2 Treatments and experimental design

Lysimeter treatments are summarised in Table 18. The experimental design consisted of 8 different treatments, with 5 replicates of each. This experiment manipulated the interactions between C and the N cycles through different pasture compositions, urine from cows on different diets and different urine-N rates. The experiment was laid out in a randomised complete block design (Appendix B).

Table 18. Treatments applied to the lysimeters and soil blocks in the second lysimeter experiment. Perennial ryegrass/white clover (PRG/WC). Perennial ryegrass/white clover/plantain (PRG/WC/P).

Treatment no.	Pasture type	Urine type	Urine rate (kg N ha^{-1})	Replicates
1	PRG/WC	PRG/WC	500	5
2	PRG/WC	PRG/WC	700	5
3	PRG/WC	PRG/WC/P	500	5
4	PRG/WC	PRG/WC/P	700	5
5	PRG/WC/P	PRG/WC	500	5
6	PRG/WC/P	PRG/WC	700	5
7	PRG/WC/P	PRG/WC/P	500	5
8	PRG/WC/P	PRG/WC/P	700	5

5.2.3 Pasture composition

As the perennial ryegrass/white clover/plantain (PRG/WC/P) pasture lysimeters/soil blocks were collected from a plantain/white clover paddock, perennial ryegrass was introduced to conform to the treatment plan (Table 18). Perennial ryegrass (cultivar 'Prospect') was sown at 20 kg ha^{-1} (0.37 g $lysimeter^{-1}$) on 7 March 2018. The perennial ryegrass was sown by cutting small rows into the soil and rubbing the seeds into these rows. Botanical dissections of lysimeter herbage were taken at each harvest. The average botanical dissection of each pasture type, over the experiment, is shown in Table 19.

Table 19. Average botanical composition of the two forages over the experiment. Perennial ryegrass/white clover (PRG/WC). Perennial ryegrass/white clover/plantain (PRG/WC/P).

	PRG/WC	PRG/WC/P
Perennial ryegrass (%)	67.9	39.6
White clover (%)	29.1	32.6
Plantain (%)	-	26.4
Dead/Weed (%)	3.0	1.5

5.2.4 Treatment application

Simulated autumn grazing

Simulated autumn grazing was performed to ensure the treatments applied to the lysimeters/soil blocks, represented typical autumn farm conditions. Simulated grazing involved harvesting of the pasture and simulated trampling, as described in Section 3.2.3. Simulated grazing was performed on 3 May 2018, after soil sampling and before urine application.

Urine

Urine collection

Urine was collected from two herds of lactating dairy cows on different diets (Table 20). The PRG/WC/P diet urine was collected on 30 April 2018, from 160 lactating Friesian/Jersey cows at the Lincoln University Dairy Farm (LUDF). The PRG/WC diet urine was collected on 1 May 2018, from 160 lactating Friesian/Jersey cows at the ADRDS. After the collection of each urine type the urine was stored in a fridge at 4 ^{o}C, until urine application.

Table 20. The diet of the two different herds of cows before urine collection. Perennial ryegrass/white clover (PRG/WC). Perennial ryegrass/white clover/plantain (PRG/WC/P).

Herd	Daily diet (22 April 2018 to 1 May 2018)
PRG/WC/P (160 cows from LUDF)	PRG/WC/P (Plantain: 25% DM) pasture (12.5 kg DM cow^{-1} day^{-1}) PRG/WC silage (6.5 kg DM cow^{-1} day^{-1}) Trace minerals – in water supply (Cu, Zn, Co, I, Se)
PRG/WC (160 cows from ADRDS)	PRG/WC pasture (9 kg DM cow^{-1} day^{-1}) Maize/Lucerne silage (50/50 mix) (8 kg DM cow^{-1} day^{-1}) Trace minerals – in water supply (Cu, Zn, Co, I, Se)

Urine standardisation

Sub-samples (50 mL) of the two urine types were analysed for nitrogen concentrations on 1 May 2018, using an Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany). The bulk urine from each herd was standardised to give the required N application rates by adding water or urea (Table 21), on 3 May 2018. Samples were taken after standardisation and analysed for N concentrations, to ensure the urine concentrations were at the required rates.

Table 21. Original urine nitrogen (N) concentration and urea/water added to standardise the urine N concentrations. Perennial ryegrass/white clover (PRG/WC). Perennial ryegrass/white clover/plantain (PRG/WC/P).

	PRG/WC/P 500 kg N ha^{-1}	PRG/WC/P 700 kg N ha^{-1}	PRG/WC 500 kg N ha^{-1}	PRG/WC 700 kg N ha^{-1}
Collected volume of urine (including excess) (L)	40	43	45	45
Urine N concentration (g N L^{-1})	6.0	6.0	5.5	5.5
Water added (L)	8.16	0	4.77	0
Urea added (g)	0	91.6	0	143.8
Final N concentration (g N L^{-1})	5.1	7.1	5.1	7.1
Final N application rate (kg N ha^{-1})	519.5	723.2	519.5	723.2

Urine application

After the urine was standardised for each treatment, 2 L of urine was applied to each lysimeter and soil block on 3 May 2018. The urine was applied as required by the treatment structure (Table 18). The urine was applied evenly over the lysimeters/soil blocks (described in Section 3.2.3) using a rosehead watering can (Figure 13). Care was taken to ensure the watering cans and measuring equipment were thoroughly washed between each urine treatment, to ensure no cross contamination occurred.

5.2.5 Measurements collected

Leachate samples were collected over the experimental period (3 May 2018 - 23 January 2019) and were analysed for NO_3^- and NH_4^+, as described in Section 3.2.4. Gas samples were collected, between 2 May 2018 and 28 August 2018, and analysed for N_2O emissions, as described in Section 3.2.4. Gas samples were collected twice weekly for the first three months, and once weekly for the final month. Herbage was collected at the 3 leaf stage or when yields were at 3000 kg DM ha^{-1}, as described for pasture management in Section 3.2.4. Soil samples were collected on days 0, 1, 7, 14, 28, 56 and 112 after urine application, as described in Section 3.2.4. These soil samples were analysed for soil mineral N and AOB *amoA* gene abundance.

5.2.6 Post treatment maintenance

Pest managment

In late June there was found to be pest damage to the plantain plants in some lysimeters/soil blocks (Figure 34). The plantain plants were severed at root level and subsequently died.

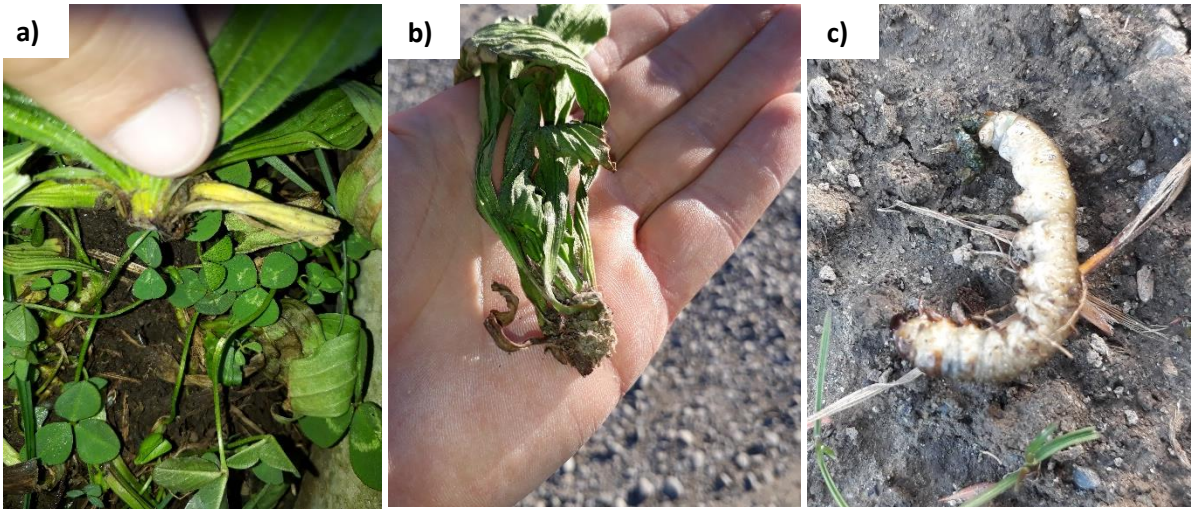


Figure 34. a) and b) Plantain plants that have pest damaged roots, and c) a dead Porina from lysimeter.

For this reason a LawnPro Lawnguard (active ingredients: 2g kg^{-1} Thiamethoxam and 0.4 g kg^{-1} Lambda-Chylothrin) was applied in granule form on 2 July 2018. Each lysimeter and soil block received 2 g (equivalent to 100 kg ha^{-1}). On 4 July 2018 dead Porina (*Wiseana cervinata*) were found located around areas of plantain damage in the lysimteres/soil blocks. Porina is a caterpillar species that is known to non-selectively sever leaves and stems at ground level, within a radius of about 50 mm from their burrow entrance (Barlow *et al*. 1986). Therefore, Porina was attributed to the plantain damage. Nevertheless the plantain content was still above 25% (Table 19).

Fertiliser application

To simulate typical farm practices, urea was applied to the lysimeters throughout the growing season. Urea was applied in four applications of 25 kg N ha^{-1}, between 18 October 2018 and 15 January 2019 (Table 22).

Table 22. Fertiliser application over the experimental period.

Product	Rate	Application date
Urea	25 kg N ha^{-1}	18 October 2018
		22 November 2018
		18 December 2018
		15 January 2019

Irrigation

A total of 265 mm of irrigation was applied to each lysimeter and soil block between 18 September 2018 and 19 January 2019 (Figure 35). Irrigation was applied using a small scale irrigation system at a rate of 5 to 10 mm per application. The irrigation scheduling simulated on farm practices to replace evapotranspiration losses and included an additional 40 mm of irrigation that was applied on 22 January 2019 to ensure the leachate breakthrough curve was complete.

5.2.7 Statistical analysis

The datasets were subject to analysis of variance (ANOVA) using Genstst (18th edition, VSN International Ltd.). For each ANOVA, orthogonal contrasts (2 x 2 x 2 factorial) were used to determine the significance of the main effect of pasture (PRG/WC vs. PRG/WC/P), urine type (PRG/WC vs. PRG/WC/P) and urine rate (500 kg N ha^{-1} vs. 700 kg N ha^{-1}). The total NH_4^+-N, NO_3^--N and mineral N leached, and AOB *amoA* gene abundance datasets were log transformed to ensure homogeneity of residual errors.

5.3 Results

5.3.1 Weather

Over the experimental period (3 May 2018 - 23 January 2019), the average daily air temperature ranged from a low of 0.8 ^oC in June 2018 to a high of 22.1 ^oC in January 2019 (Figure 35a). Daily average soil temperature (100 mm depth) ranged from a low of 4.0 ^oC in June 2018 to a high of 23.5 ^oC in January 2019 (Figure 35a). Temperatures followed expected cyclical trends, with warmer temperatures during summer and cooler temperatures during winter. Water inputs over the 8 and half months experimental period totalled 691 mm; comprising 415 mm of rainfall, 265 mm of irrigation and 11 mm of water input from treatments. The rainfall (415 mm) was lower than the average rainfall (473 mm) for this period (Average based of long term average. 1971-2000, Lincoln weather station 4881).

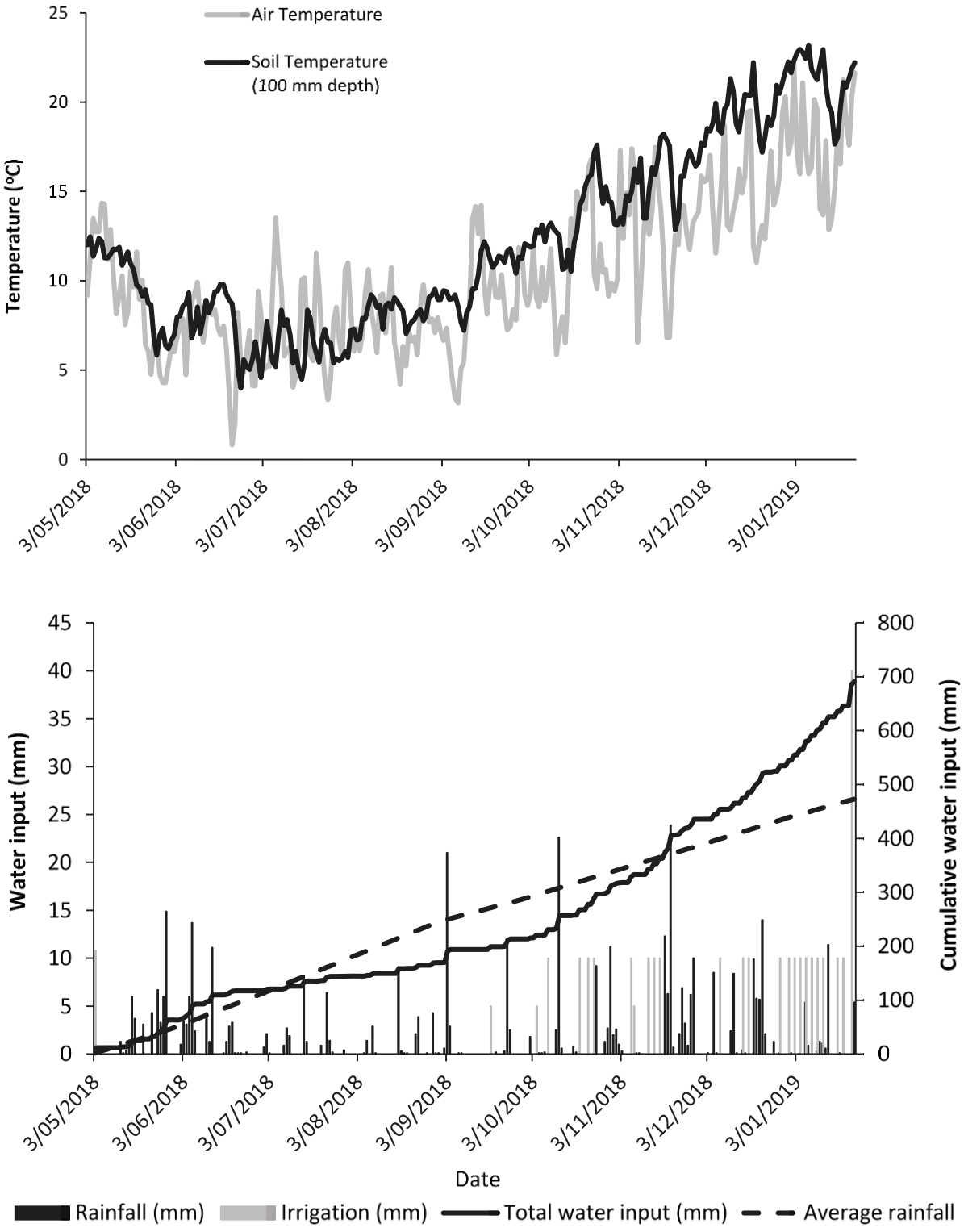


Figure 35.a) Average daily air temperature and soil temperature (at 100 mm depth), and b) daily rainfall, irrigation water inputs and cumulative water input over the experimental period (3 May 2018 - 23 January 2019), and long-term average rainfall data (1971-2000, Lincoln weather station 4881).

5.3.2 Drainage

The effect of each treatment on drainage is shown in Figure 36. The total average amount of drainage ranged from 133 to 179 mm. The PRG/WC pasture had significantly less drainage than the PRG/WC/P pasture ($P < 0.001$). Urine type ($P = 0.055$) and urine rate ($P = 0.291$) had no significant effect on drainage.

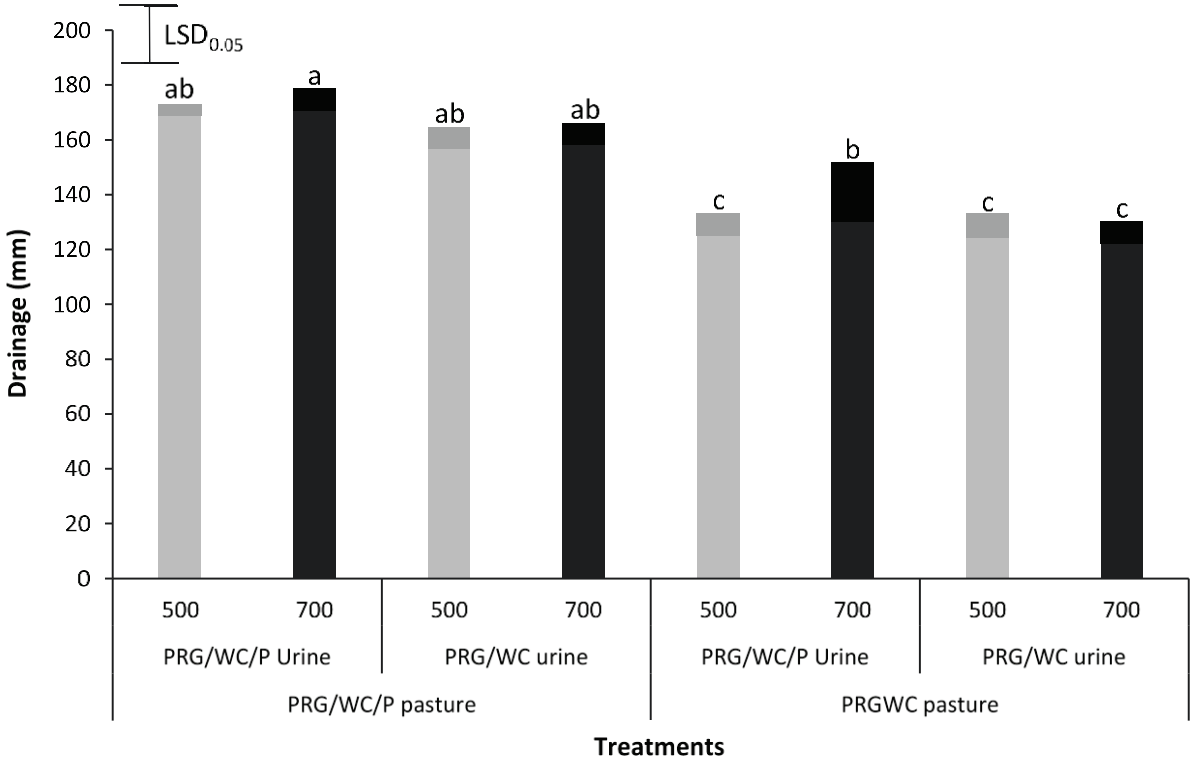


Figure 36. Total average drainage (mm) over the experiment period (3 May 2018 - 23 January 2019) under each treatment. Least significant difference (LSD) is at the 5% level (*n* = 5). LSD = 26.75. Bars with a letter in common are not significantly different at the 5% level. Perennial ryegrass/white clover/plantain (PRG/WC/P). Perennial ryegrass/white clover (PRG/WC). 500 = 500 kg N ha^{-1}. 700 = 700 kg N ha^{-1}.

5.3.3 Nitrogen leaching

A complete NO_3^- leaching breakthrough curve was obtained, for each treatment (Figure 37), with peak NO_3^- concentration values ranging from 35.7 to 439.4 mg N L^{-1}. The NO_3^- concentration peaks occurred between 60 to 70 mm of cumulative drainage.

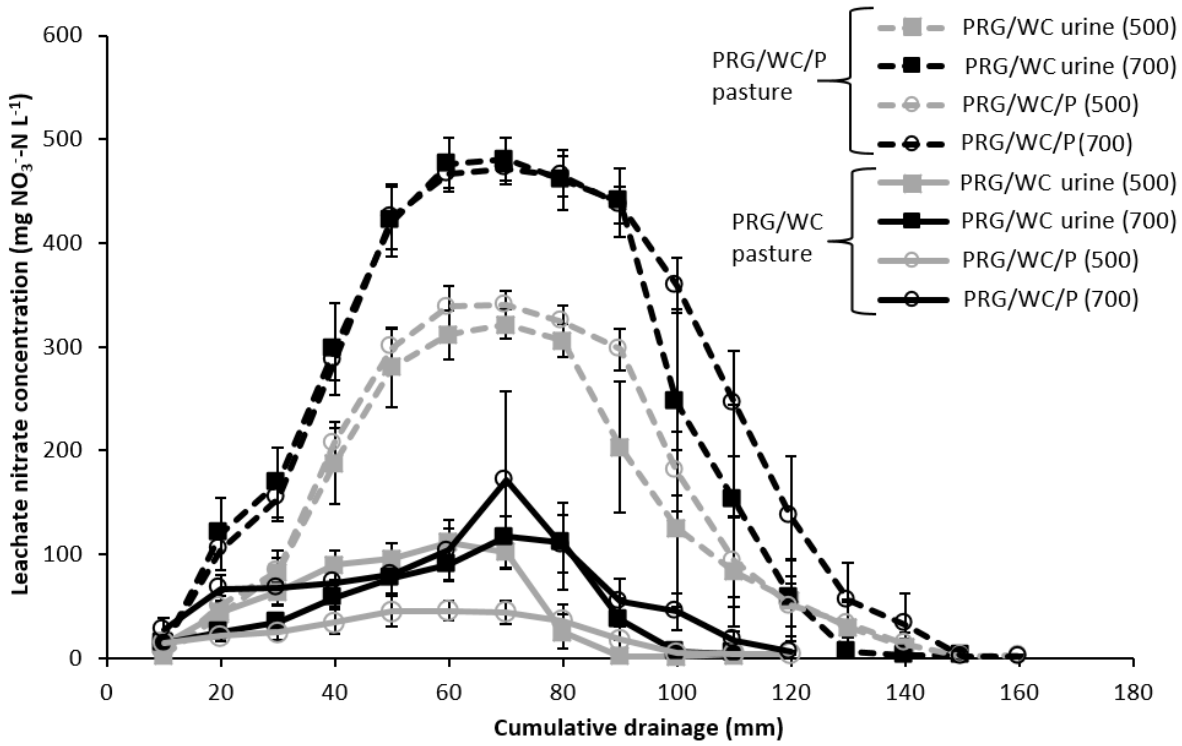


Figure 37. The average nitrate concentration in leachate plotted against cumulative drainage (mm) over the sampling period (3 May 2018 - 23 January 2019) under each treatment. Error bars are standard error of the mean (*n* = 5). Perennial ryegrass/white clover/plantain (PRG/WC/P). Perennial ryegrass/white clover (PRG/WC). 500 = 500 kg N ha^{-1}. 700 = 700 kg N ha^{-1}.

The effects of each treatment on total log NH_4^+-N, NO_3^--N and mineral N leached over the sampling period (3 May 2018 - 23 January 2019) are shown in Table 23. The PRG/WC pasture leached significantly less NO_3^--N than the PRG/WC/P pasture (P < 0.001). The urine application rate of 500 kg N ha^{-1} leached significantly less NO_3^--N than the 700 kg N ha^{-1} urine application rate (P < 0.001). Urine type had no significant effect (P = 0.473) on total NO_3^--N leached.

The urine rate of 500 kg N ha^{-1} had significantly lower amount of total NH_4^+-N leached than the urine rate of 700 kg N ha^{-1} (P < 0.001). Pasture type (P = 0.437) and urine type (P = 0.337) had no significant effect on total NH_4^+-N leached.

The PRG/WC pasture leached significantly less mineral N than the PRG/WC/P pasture (P < 0.001). The urine rate of 500 kg N ha^{-1} leached significantly less mineral N than the 700 kg N ha^{-1} urine rate (P < 0.001). Urine type had no significant effect (P = 0.947) on total mineral N leached.

Table 23. The effect each treatment had on total log nitrate, log ammonium and log mineral N leached over the sampling peroid (3 May 2018 - 23 January 2019). The contrasts are main effect contrasts for a 2 x 2 x 2 factorial, with factors pasture (PRG/WC vs PRG/WC/P), urine type (PRG/WC vs PRG/WC/P) and urine rate (500 kg N ha vs 700 kg N ha). Perennial ryegrass/white clover/plantain (PRG/WC/P). Bars with a letter in common are not significantly different at the 5% level. Perennial ryegrass/white clover (PRG/WC).

		Log_{10} means (Brackets contain back transformed mean)					
Pasture	**Urine application type and rate (kg N ha^{-1})**	**Ammonium leached (kg NH_4^+-N ha^{-1})**		**Nitrate leached (kg NO_3^--N ha^{-1})**		**Mineral N leached (kg N ha^{-1})**	
PRG/WC	PRG/WC (500)	1.134	**(13.6)^{cd}**	1.581	**(38.1)^{d}**	1.725	**(53.1)^{d}**
	PRG/WC (700)	1.210	**(16.2)^{bcd}**	1.582	**(38.2)^{d}**	1.740	**(55.0)^{cd}**
	PRG/WC/P (500)	1.012	**(10.3)^{d}**	1.253	**(17.9)^{e}**	1.465	**(29.2)^{e}**
	PRG/WC/P (700)	1.438	**(27.4)^{abc}**	1.686	**(48.5)^{d}**	1.887	**(77.1)^{c}**
PRG/WC/P	PRG/WC (500)	0.920	**(8.3)^{d}**	2.278	**(189.7)^{c}**	2.301	**(200.0)^{b}**
	PRG/WC (700)	1.502	**(31.8)^{ab}**	2.475	**(298.5)^{ab}**	2.523	**(333.4)^{a}**
	PRG/WC/P (500)	1.069	**(11.8)^{d}**	2.339	**(218.3)^{bc}**	2.364	**(231.2)^{b}**
	PRG/WC/P (700)	1.543	**(34.9)^{a}**	2.516	**(328.1)^{a}**	2.563	**(365.6)^{a}**
LSD (5%) (*n* = 5)		0.311		0.172		0.153	
P value for contrasts	Pasture	0.437		<0.001		<0.001	
	Urine type	0.337		0.473		0.947	
	Urine rate	<0.001		<0.001		<0.001	

5.3.4 Nitrous oxide emissions

The effect of each treatment on total N_2O-N emissions over the sampling period (2 May 2018 – 28 August 2018) is shown in Figure 38. The PRG/WC pasture had significantly lower N_2O-N emissions than the PRG/WC/P pasture (*P* <0.001). The 500 kg N ha^{-1} urine rate had significantly lower total N_2O-N emissions over the sampling period, than the 700 kg N ha^{-1} urine rate (*P* < 0.001). Urine type had no significant effect on total N_2O-N emissions over the sampling period (*P* = 0.338).

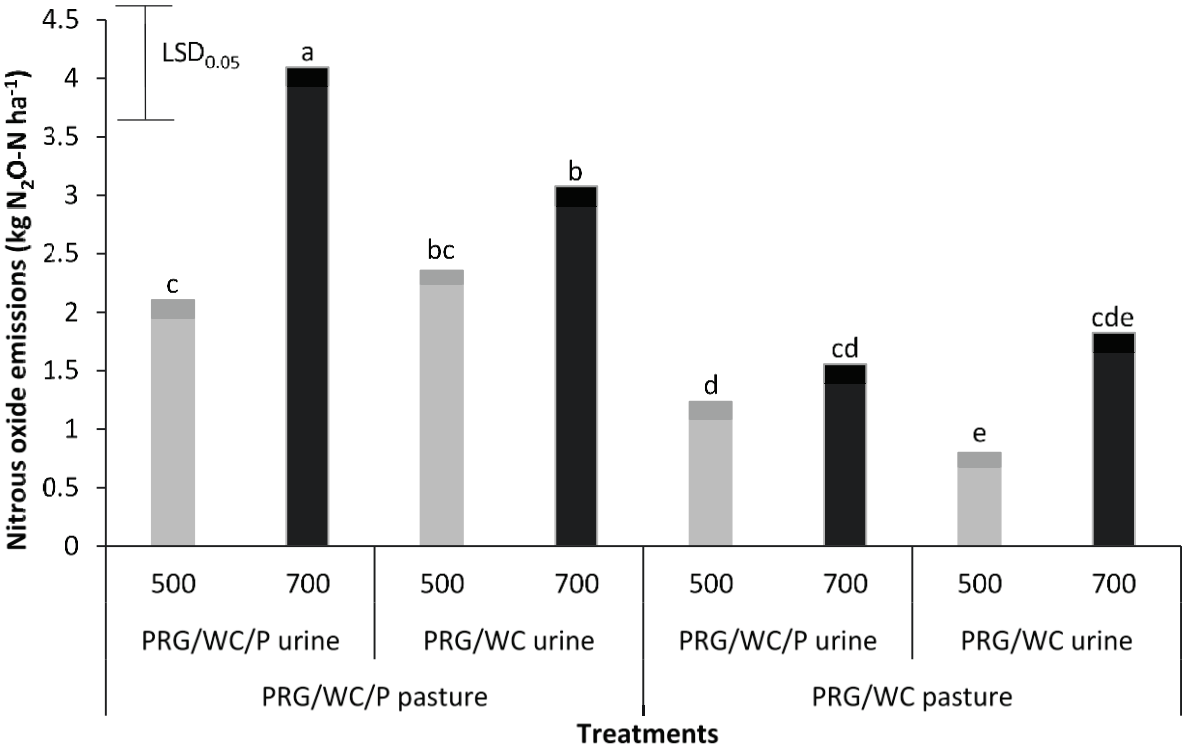


Figure 38. The effect of different treatments on nitrous oxide emissions over the sampling period (2 May 2018 - 28 August 2018). Least significant difference (LSD) error bar is at the 5% level. LSD = 0.977. Bars with a letter in common are not significantly different at the 5% level. Perennial ryegrass/white clover/plantain (PRG/WC/P). Perennial ryegrass/white clover (PRG/WC). 500 = 500 kg N ha^{-1}. 700 = 700 kg N ha^{-1}.

5.3.5 Herbage

Experimental yield and nitrogen uptake

The effect each treatment had on yield and plant N uptake over the experimental period (3 May 2018 - 23 January 2019) is shown in Figure 39. The PRG/WC pasture had significantly higher yield ($P < 0.001$) and plant N uptake ($P < 0.001$) than the PRG/WC/P pasture. Urine type had no significant effect on yield ($P = 0.780$) or plant N uptake ($P = 0.750$). Urine rate had no significant effect on yield ($P = 0.111$) or plant N uptake ($P = 0.055$).

Winter yield and nitrogen uptake

The effect of each treatment on yield and plant N uptake over the winter period (3 May 2018-17 September 18) is shown in Figure 40. The PRG/WC pasture had significantly higher winter yield ($P < 0.001$) and plant N uptake ($P < 0.001$) than the PRG/WC/P pasture. Urine type had a significant effect on winter pasture yield ($P = 0.044$) and plant N uptake ($P < 0.039$), with the PRG/WC urine having significantly higher winter yield and plant N uptake than the PRG/WC/P urine. Urine rate had no significant effect on winter yield ($P = 0.885$) and plant N uptake ($P = 0.060$).

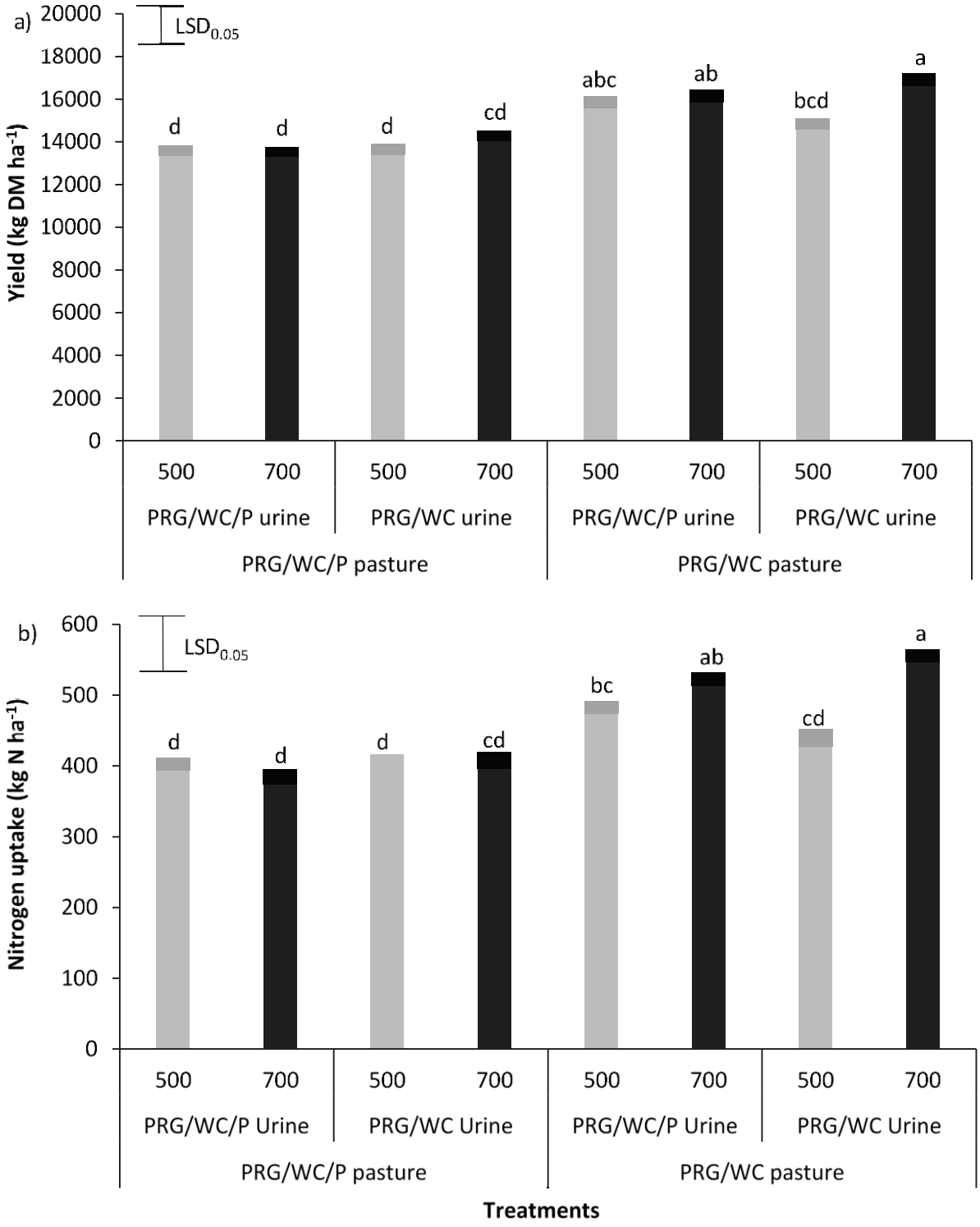


Figure 39. The effect of each treatment effect on a) pasture yield and b) over the experimental period (3rd May 2018 - 23rd January 2019). Least significant difference (LSD) is at the 5% level. a) LSD = 1856.3. b) LSD = 72.4. Bars with a letter in common are not significantly different at the 5% level. Perennial ryegrass/white clover/plantain (PRG/WC/P). Perennial ryegrass/white clover (PRG/WC). 500 = 500 kg N ha^{-1}. 700 = 700 kg N ha^{-1}.

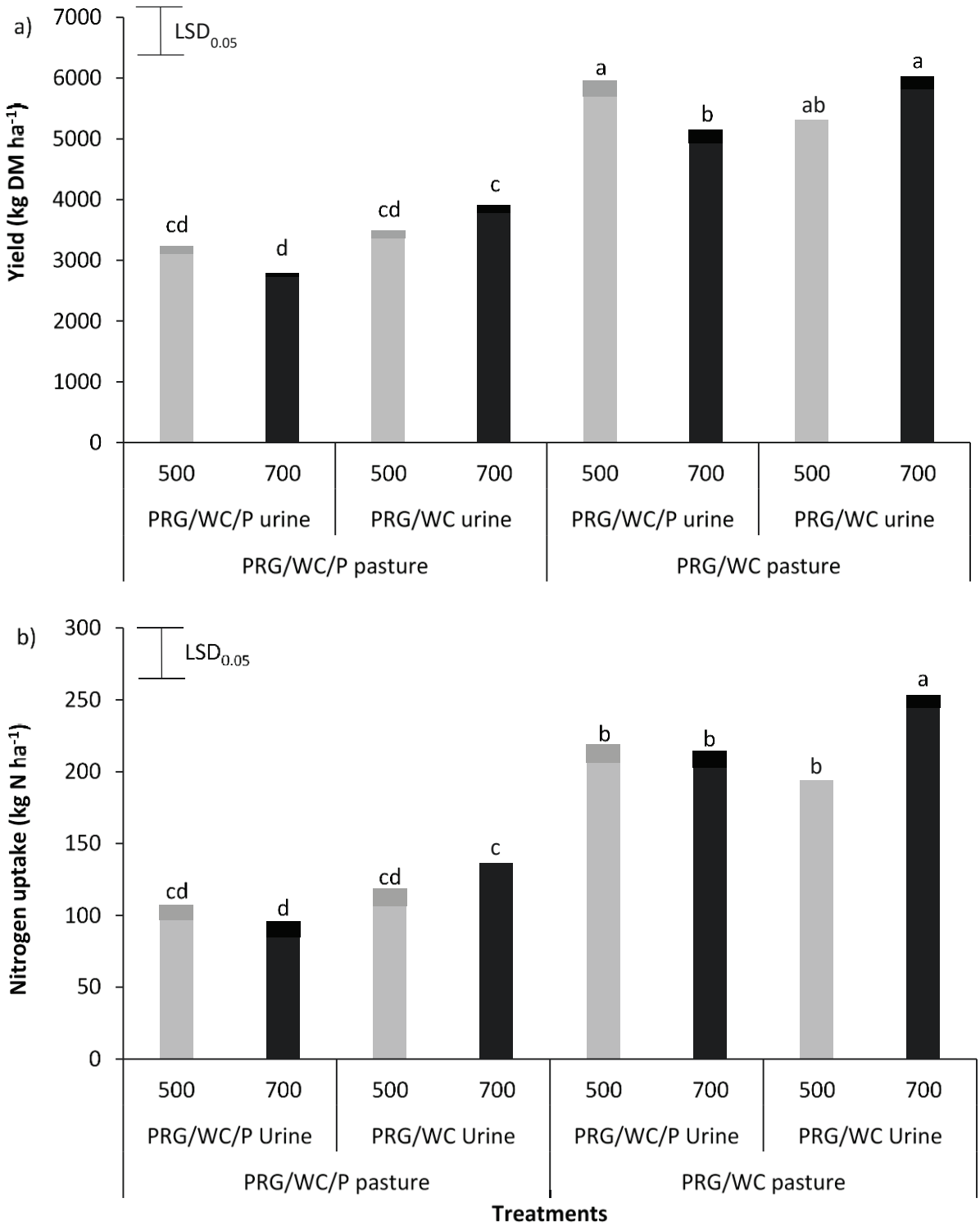


Figure 40. The effect of each treatment on a) pasture yield and b) plant N uptake over the winter period (3 May 2018 – 17 September 2018). Least significant difference (LSD) is at the 5% level. a) LSD = 784.1. b) LSD = 31.7. Bars with a letter in common are not significantly different at the 5% level. Perennial ryegrass/white clover/plantain (PRG/WC/P). Perennial ryegrass/white clover (PRG/WC). 500 = 500 kg N ha^{-1}. 700 = 700 kg N ha^{-1}.

5.3.6 Soils

Mineral N

The effect of pasture, urine type and urine N rate on total soil mineral N (NO_3^--N + NH_4^+-N) over the first 112 days after urine application is shown in Figure 41. Using the area under the curve (AUC) for the first 112 days after urine application it was shown that the PRG/WC pastures had significantly lower average soil mineral N than the PRG/WC/P pasture ($P < 0.001$). The urine rate of 500 kg N ha^{-1} had significantly lower average soil mineral N than the 700 kg N ha^{-1} urine rate ($P < 0.001$). Urine type had no significant effect ($P = 0.420$).

AOB gene abundance

The effect of each treatment on AOB *amoA* gene abundance over the first 112 days after urine application is shown in Figure 42. Using the log transformed AUC for the first 112 days after urine application, pasture type ($P < 0.001$) and urine rate ($P = 0.003$) were shown to have a significant effect on AOB *amoA* gene abundance. The PRG/WC pasture had significantly lower AOB *amoA* gene abundance than the PRG/WC/P pasture. The urine rate of 500 kg N ha^{-1} had significantly lower AOB *amoA* gene abundance than the 700 kg N ha^{-1} urine rate. Urine type had no significant effect on average AOB *amoA* gene abundance ($P = 0.947$).

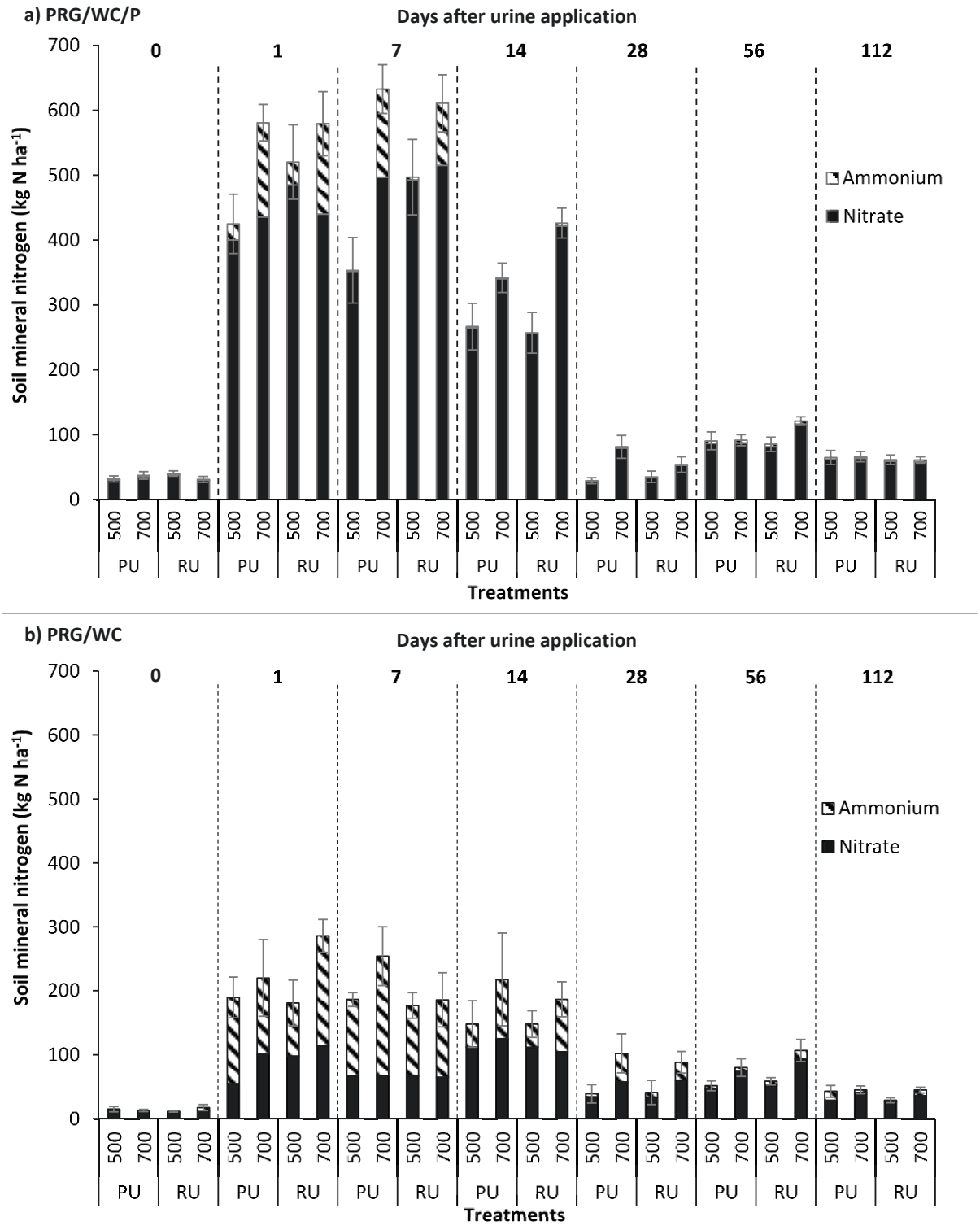


Figure 41. The effect of each urine treatment on soil mineral N (NO_3^--N + NH_4^+-N) over the first 112 days after urine application, beneath a) Perennial ryegrass/white clover/plantain (PRG/WC/P) and b) Perennial ryegrass/white clover (PRG/WC). Samples were taken from 0-100 mm depth in the soil profile. Error bars are standard error of the mean (*n* = 5). PU = PRG/WC/P urine. RU = PRG/WC urine. 500 = 500 kg N ha^{-1} urine application rate. 700 = 700 kg N ha^{-1} urine application rate.

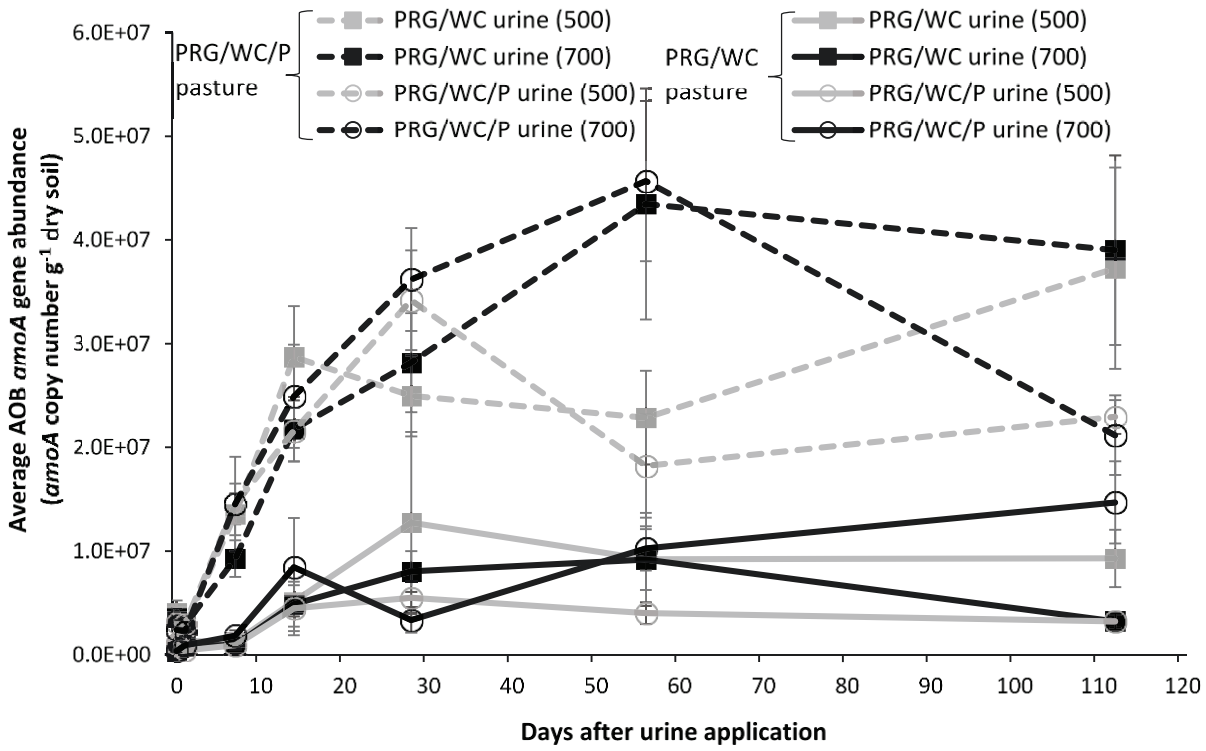


Figure 42. The effect of pasture, urine type and urine nitrogen (N) rate on soil AOB *amoA* gene copies over the first 112 days after urine application. Samples were taken from 0-100 mm depth in the soil profile. Error bars are standard error of the mean (*n* = 5). Perennial ryegrass/white clover/plantain (PRG/WC/P). Perennial ryegrass/white clover (PRG/WC). 500 = 500 kg N ha^{-1}. 700 = 700 kg N ha^{-1}.

5.4 Discussion

5.4.1 Pasture type

The PRG/WC pasture had significantly higher total yield ($P < 0.001$) and winter yield ($P < 0.001$), than the PRG/WC/P pasture. The higher yield under the PRG/WC pasture, compared with the PRG/WC/P pasture, is likely due to the better establishment of the perennial ryegrass. This higher yield and subsequently higher plant N uptake ($P < 0.001$), resulted in the lower soil mineral N ($P < 0.001$). The lower soil mineral NH_4^+-N under the PRG/WC resulted in lower AOB *amoA* gene abundance; as soil NH_4^+ is the energy source for AOB. The lower soil mineral N and AOB *amoA* gene abundance resulted in a smaller amount of soil NO_3^--N available to be leached from the PRG/WC pasture and therefore resulted in a lower amount of NO_3^--N leached under the PRG/WC pasture, compared with PRG/WC/P pasture (Figure 37). The drainage volume was also significantly lower under the PRG/WC pasture, compared with the PRG/WC/P pasture ($P < 0.001$). This lower amount of drainage volume is likely due to the higher yield (and higher transpiration) under the PRG/WC pasture, compared with the PRG/WC/P pasture. The combination of lower drainage volume and lower leachate NO_3^--N concentration resulted in the lower total NO_3^--N leached under PRG/WC pasture ($P < 0.001$). Averaged over all the treatments, the PRG/WC pasture leached 87% less NO_3^--N than the PRG/WC/P pasture.

The lower soil mineral N and AOB *amoA* gene abundance in the PRG/WC treatment also resulted in the significantly lower N_2O-N emissions, as there is lower soil NO_3^--N available to undergo denitrification under the PRG/WC pasture, compared with the PRG/WC/P pasture. Averaged over all the treatments, the PRG/WC pasture had 53% lower N_2O-N emissions than the PRG/WC/P pasture.

These lower amounts of NO_3^--N leached and N_2O-N emissions under the PRG/WC, compared with PRG/WC/P, can be attributed to the large difference in yield. This yield difference, and subsequently a reduction in N losses, is due to the perennial ryegrass being better established in the PRG/WC pasture than the PRG/WC/P pasture; and the plantain damage in the PRG/WC/P pasture (described in Section 5.2.6).

Due to the inconsistency in perennial ryegrass yield and the porina damage to the plantain, these results are not a fair comparison between PRG/WC and PRG/WC/P pastures. Further research is needed to compare the effects equally established PRG/WC and PRG/WC/P pastures have on N losses.

5.4.2 Urine rate

The 500 kg N ha^{-1} urine rate had significantly lower ($P < 0.001$) average soil mineral N content than the 700 kg N ha^{-1} urine rate (Figure 41). This lower soil mineral N content is due to the lower N input from the 500 kg N ha^{-1} urine rate compared with the 700 kg N ha^{-1} urine rate. This lower soil mineral N, led to significantly lower ($P = 0.003$) AOB *amoA* gene abundance (Figure 42). The lower soil mineral N and AOB *amoA* gene abundance, meant that there was less NO_3^--N and NH_4^+ available to be leached or denitrified, resulting in lower N leaching losses (Table 23) and N_2O emissions (Figure 38). The difference in N leaching ranged from 3% to 62%. Averaged over all the treatments, the N leaching losses from the 500 kg N ha^{-1} urine rate were 39% lower than the 700 kg N ha^{-1} urine rate. The reductions in N_2O emission under the 500 kg N ha^{-1} urine rate were 21% to 56% lower than the 700 kg N ha^{-1} urine rate. Averaged over all the treatments, the N_2O emissions from the 500 kg N ha^{-1} urine rate were 38% lower than from the 700 kg N ha^{-1} urine rate.

Literature suggests that reducing the urine-N rate is an efficient way of reducing N leaching losses (Di & Cameron 2007; Malcolm *et al*. 2015a; Woods *et al*. 2018). In a lysimeter study, with urine applied to Italian ryegrass/white clover pasture in March, Woods *et al*. (2018) showed that by reducing the urine-N rate from 700 kg N ha^{-1} to 508 kg N ha^{-1}, N leaching losses were reduced by 79.8%. In a lysimeter study, with urine applied to winter grazed kale in June, Malcolm *et al*. (2015a), showed that by reducing the urine-N rate from 700 kg N ha^{-1} down to 500 kg N ha^{-1}, N leaching losses were reduced by 43.9%. In a lysimeter study, with urine applied to PRG/WC pasture in May, Di and Cameron (2007) showed that by reducing the urine-N rate from 700 kg N ha^{-1} to 300 kg N ha^{-1}, N leaching losses were reduced

by 68.2%. The literature is consistent with the findings of this study, which suggest reducing urine-N loading rates is an effective way of reducing N leaching losses.

Literature suggests reducing urine-N can also lead to significant reductions in N_2O emissions (Singh *et al*. 2009; Kelliher *et al*. 2014; Selbie *et al*. 2014). In a two year lysimeter experiment, Selbie *et al*. (2014) showed that reducing the urine-N rate from 700 kg N ha^{-1} to 500 kg N ha^{-1} reduced N_2O emissions by 53% and 56%, in year 1 and 2 of the experiment, respectively. In a soil core study performed in glasshouse conditions, Singh *et al*. (2009) showed that by reducing the urine-N rate from 570 kg N ha^{-1} to 290 kg N ha^{-1}, N_2O emissions were reduced by 84%. Kelliher *et al*. (2014), found a linear relationship between N application rate and N_2O emissions; using the linear regression provided by this study, the reduction in urine-N rate from 700 kg N ha^{-1} to 500 kg N ha^{-1} is estimated to reduced N_2O emissions by 26%. The findings of this study are consistent with literature that suggests reducing the urine-N loading rate can lead to significant reductions in N_2O emissions.

The findings of this study, together with supporting literature, show the potential of reducing urine-N rate as a mitigation tool to reduce N losses in Canterbury soils. Reducing the urine-N loading rate by 28% (from 700 kg N ha^{-1} to 500 kg N ha^{-1}), resulted in a 38% reduction in N_2O emissions and a 39% reduction in N leaching losses. More research is needed to help develop effective and economic strategies that enable farmers to reduce cattle urine-N loading rates.

5.4.3 Urine type

Urine type had no significant effect on soil mineral N (P = 0.420), AOB *amoA* gene abundance (P = 0.947), N leaching losses (P = 0.947) and N_2O emissions (P = 0.338). Incorporating plantain (16%) into the cattle diet, appears to be no significant effect on N transformations and losses. This is in contrast with the some previous literature which suggests plantain urine may have a BNI effect (Judson *et al*. 2018).

5.5 Conclusions

- The comparison of the PRG/WC pasture with PRG/WC/P was heavily influenced by the large difference in yield. Yield difference was due to the perennial ryegrass being better established in the PRG/WC pasture compared to the PRG/WC/P pasture, and the plantain damage by the porina in the PRG/WC/P pasture. For this reason any comparison of pasture types in this experiment would be unreliable. Further research is needed to compare the effects equally established PRG/WC and PRG/WC/P pastures have on N transformations/losses under shallow stony soils (this is the aim of the next chapter).

- Lowering the urine-N loading rate by 28%, from 700 kg N ha^{-1} to 500 kg N ha^{-1}, produced a 38% reduction in N_2O emissions and a 39% reduction in total N leaching losses. This confirms that reducing urine-N loading rate could be an effective way of reducing N losses from grazed pasture systems.

- Urine type had no effect on N transformations or N losses. Further research is needed to determine the effect of plantain at a higher proportion than 16% of the diet.

Chapter 6

Lysimeter experiment 3

6.1 Introduction

Traditional grazed pastoral systems in temperate climates have been dominated by perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens L*.) (PRG/WC) pastures. However, diets consisting of traditional PRG/WC pastures result in large excesses of N for the cattle consuming it. Excess N is excreted at high rates (approximately 700 kg N ha^{-1}) onto soil, via cattle urine patches (Selbie *et al*. 2015). The high rate of N deposited in urine patches often exceeds plant requirements, especially in autumn and winter months when plant growth and N uptake is limited. Surplus N can then be lost to the wider environment through NO_3^- leaching, causing surface water eutrophication and elevated NO_3^- concentrations in drinking water supplies. High NO_3^- concentrations (i.e. >11.3 mg L^{-1} NO_3^--N) in drinking water present a human health risk. There is also a significant economic cost associated with lost N, as it must be replaced by fertilisers. It is therefore necessary to improve understanding of the factors that affect agricultural N leaching losses and develop effective strategies to reduce losses.

Sowing plantain (*Plantago lanceolate* L.) into traditional pastures has been proposed as a potential tool for reducing N leaching (Romera *et al*. 2017; Carlton *et al*. 2018; Woods *et al*. 2018; Welten *et al*. 2019). It has also been suggested that plantain can produce a biological nitrification inhibitor (BNI) effect in soil (Dietz *et al*. 2013; Carlton *et al*. 2018). However, there has been limited research on the effect of plantain on N losses from urine applied over a range of dates onto shallow stony soils.

Italian ryegrass (*Lolium multiforum Lam*.) has also been proposed as a potential option for reducing N leaching from farms (Moir *et al*. 2013; Malcolm *et al*. 2014; Woods *et al*. 2016; Maxwell *et al*. 2018), as winter growth in Italian ryegrass (IRG) is significantly more winter active than growth in PRG (Kemp 1999; Charlton & Stewart 2000). However, there has been no research on the effect of IRG on N losses from urine applied over a range of dates onto shallow stony soils.

Therefore the objectives of this lysimeter experiment were to quantify the effects of plantain and IRG on N leaching losses from urine-treated soil, and the effect on the key soil processes of immobilisation and nitrification (including ammonia oxidising bacteria (AOB) abundance), over a range of urine application dates, on shallow stony soils.

6.2 Research article

This section has been submitted to Soil Use and Management, January 2020.

Effect of cattle urine deposition timing and pasture composition on nitrogen leaching losses

Abstract

Nitrogen (N) losses from agricultural land is a major environmental concern, as N leaching can cause eutrophication of waterways. A lysimeter experiment with undisturbed monoliths from a stony silt loam soil extracted from a field site, was carried out to quantify the effects of incorporating plantain and Italian ryegrass into pasture on N losses from urine applied to soil on different dates. Three pasture types were tested: (i) perennial ryegrass (*Lolium perenne*)/white clover (*Trifolium repens*) (PRG/WC), (ii) perennial ryegrass/white clover/plantain (*Plantago lanceolate*) (PRG/WC/P), and (iii) Italian ryegrass (*Lolium multiforum*)/white clover/plantain (IRG/WC/P), over four different urine applications dates (February - late summer, March - early autumn, April - autumn, and May - late autumn). Cattle urine was applied at the equivalent of 700 kg N ha^{-1}. There was a significant reduction in total amount of nitrate (NO_3^--N) leached under the PRG/WC/P (14% reduction) and IRG/WC/P (24% reduction) compared with the PRG/WC. The reductions were attributed to an increase in plant N uptake, which decreased the soil mineral N content and subsequently reduced the amount of N available to be leached; and an increase in herbage yield which increased transpiration, thus reducing drainage volume.

Introduction

Traditional grazed pastoral systems in temperate climates have been dominated by perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens L*.) (PRG/WC) pastures. However, diets consisting of traditional PRG/WC pastures result in large excesses of nitrogen (N) for the cattle consuming it. Excess N is excreted at high rates (approximately 700 kg N ha^{-1}) onto soil, via cattle urine patches (Selbie *et al*. 2015). The high rate of N deposited in urine patches often exceeds plant requirements, especially in autumn and winter months when plant growth and N uptake is limited. Surplus N can then be lost to the wider environment through nitrate (NO_3^-) leaching. When drainage occurs, NO_3^- can leach through the soil profile and enter surface water and groundwater, causing surface water eutrophication and elevated NO_3^- concentrations in drinking water supplies. High NO_3^- concentrations (i.e. >11.3 mg L^{-1} NO_3^--N) in drinking water present a human health risk. There is also a significant economic cost associated with lost N, as it must be replaced by fertilisers. It is therefore necessary to improve understanding of the factors that affect agricultural N leaching losses and develop effective strategies to reduce losses.

Sowing plantain (*Plantago lanceolate* L.) into traditional pastures has been proposed as a potential tool for reducing N leaching (Romera *et al*. 2017; Carlton *et al*. 2018; Welten *et al*. 2019). It has also been suggested that plantain can produce a biological nitrification inhibitor (BNI) effect in soil (Dietz *et al*. 2013; Carlton *et al*. 2018). Lysimeter studies have shown reductions in N leaching from urine applied to pastures containing plantain, compared to pastures without plantain. Carlton *et al*. (2018) found 82% and 74% reductions in mineral N leaching from perennial ryegrass/white clover with plantain (PRG/WC/P), compared with traditional PRG/WC, when urine (700 kg N ha^{-1}) was applied in December and February, respectively. Woods *et al*. (2018) found a pasture mix of Italian ryegrass, white clover and plantain (IRG/WC/P) leached 46% less mineral N than PRG/WC, when urine (700 kg N ha^{-1}) was applied in March. Welten *et al*. (2019) found 15%, 27% and 52% reductions in mineral N leaching from pure plantain swards compared with pure PRG swards, for summer, autumn and winter applied urine (622 kg N ha^{-1}), respectively. However, there has been no research on the effect of plantain on N losses from urine applied over a range of dates onto shallow stony soils.

Italian ryegrass (*Lolium multiforum Lam*.) has also been proposed as a potential option for reducing N leaching from farms, as winter growth in Italian ryegrass (IRG) is significantly more winter active than growth in PRG (Kemp 1999; Charlton & Stewart 2000). In a glasshouse study, Moir *et al*. (2013) found two IRG cultivars 'Feast 2' (134 kg N ha^{-1}) and 'Tama' (130 kg N ha^{-1}) leached less N than two PRG cultivars 'Aber Magic' (280 kg N ha^{-1}) and 'Alto' (310 kg N ha^{-1}), when urine (700 kg N ha^{-1}) was applied. Malcolm *et al*. (2014) conducted a lysimeter study, which compared N leaching losses under IRG/WC and PRG/WC, where urine (1000 kg N ha^{-1}) was applied in May (late autumn). It was found that IRG/WC leached 24-25% less N than PRG/WC. Woods *et al*. (2016) found in a lysimeter study that pure IRG leached 35% less N than PRG/WC, under a urine patch (700 kg N ha^{-1}) applied in May. Maxwell *et al*. (2018) conducted a lysimeter study, which compared N leaching losses under PRG against IRG, when urine (700 kg N ha^{-1}) was applied in April (autumn). This study found that IRG leached 33-46% less N than the PRG. However, there has been no research on the effect of IRG on N losses from urine applied over a range of dates onto shallow stony soils.

Therefore the objectives of this study were to quantify the effects of plantain and IRG on N leaching losses from urine-treated soil, and the effect on the key soil processes of immobilisation and nitrification (including ammonia oxidising bacteria (AOB) abundance), over a range of urine application dates, on shallow stony soils.

Materials and methods

Lysimeter and soil block collection and installation

The experiment was conducted at Lincoln University's Ashley Dene Research and Development Station (ADRDS), near Lincoln, Canterbury (43°38'51.2"S 172°20'45.5"E; 17 m above sea level). For this

experiment, 60 undisturbed soil monolith lysimeters, 500 mm in diameter and 700 mm deep, were used. The lysimeter collection area was located in a plantain (*Plantago lanceolate* L.) cv. 'Tonic' and white clover (*Trifolium repens* L.) cv. 'Legacy' paddock (43°38'42.9"S 172°20'51.0"E). The collection site was divided into two adjoining areas; one area sown with perennial ryegrass (*Lolium perenne* L.) cv. 'Prospect' seed (15 kg ha^{-1}) and the other area sown with Italian ryegrass (*Lolium multiflorum* Lam) cv. 'Tabu' seed (25 kg ha^{-1}). Both areas were sown in November (late spring) 2018. Of the 60 lysimeters, 20 were collected from the Italian ryegrass (IRG) area and 40 were collected from the perennial ryegrass (PRG) area. Of the 40 lysimeters collected from the PRG area, plantain was removed from 20 in December (early summer) 2018, to ensure the correct plant species compositions (Table 24). Herbage samples were botanically dissected throughout the experiment and the average compositions of each pasture type are shown in Table 25.

Table 24. Treatments applied to the lysimeters and soil blocks. Perennial ryegrass/white clover (PRG/WC). Perennial ryegrass/white clover/plantain (PRG/WC/P). Italian ryegrass/white clover/plantain (IRG/WC/P).

Treatment number	Pasture type	Urine application (Month)	Urine rate (kg N ha^{-1})	Replicates	Measurement period
1	PRG/WC	February	700	5	209 days (20 February – 16 September)
2	PRG/WC/P	February	700	5	
3	IRG/WC/P	February	700	5	
4	PRG/WC	March	700	5	181 days (20 March – 16 September)
5	PRG/WC/P	March	700	5	
6	IRG/WC/P	March	700	5	
7	PRG/WC	April	700	5	153 days (16 April – 16 September)
8	PRG/WC/P	April	700	5	
9	IRG/WC/P	April	700	5	
10	PRG/WC	May	700	5	125 days (14 May – 16 September)
11	PRG/WC/P	May	700	5	
12	IRG/WC/P	May	700	5	

Table 25. Average botanical composition of the three pasture types, over the measurement period (20 February 2019 – 16 September 2019). Perennial ryegrass/white clover (PRG/WC). Perennial ryegrass/white clover/plantain (PRG/WC/P). Italian ryegrass/white clover/plantain (IRG/WC/P).

	PRG/WC	PRG/WC/P	IRG/WC/P
Perennial ryegrass (%)	54.1	32.4	-
Italian ryegrass (%)	-	-	52.1
White clover (%)	44.0	31.1	20.2
Plantain (%)	-	35.0	26.3
Weed + Dead (%)	1.9	1.5	1.4

The lysimeters were collected in November (late spring) 2018 following well-established protocols and procedures (Cameron *et al*. 1992). The lysimeters were installed into a lysimeter trench facility at ADRDS and were maintained to ensure they replicated typical paddock growing conditions. Each lysimeter had a corresponding soil block, 500 mm in diameter and 200 mm deep, installed next to it, to allow the periodic removal of soil samples (Malcolm *et al*. 2019; Talbot *et al*. 2019). The soil blocks were collected from the same location as their corresponding lysimeters and installed into the lysimeter/soil block facility one week before urine application.

The soil in all of the lysimeters and soil blocks was a Lismore/Balmoral stony silt loam (Pallic Firm Brown soil) (Hewitt 2010), Udic Haplustept (Soil Survey Staff. 2014) (Table 4). This soil is very stony, shallow, free-draining and typical of soils in the Canterbury region of New Zealand. Soil samples were collected from 0-75 mm depth for basic soil analysis at Analytical Research Laboratories (Ravensdown, New Zealand) (Table 26).

Table 26. Results of the basic soil analysis performed at the lysimeter collection site at 0-75 mm depth. Cation exchange capacity (CEC). Exchangeable (Exc.).

pH	6.2
Olsen P (µg g^{-1})	17
Sulphate Sulphur (µg g^{-1})	3
CEC ($cmol_c$ kg^{-1})	19.3
Exc. Ca ($cmol_c$ kg^{-1})	11.7
Exc. Mg ($cmol_c$ kg^{-1})	0.9
Exc. K ($cmol_c$ kg^{-1})	1.1
Exc. Na ($cmol_c$ kg^{-1})	0.3

Basal superphosphate fertiliser (8.2 g $lysimeter^{-1}$ equivalent to 444 kg P ha^{-1}) was applied to each lysimeter and soil block on 6 February 2019. This basal fertiliser was applied because the Olsen P (17 µg g^{-1}) and sulphate super values (3 µg g^{-1}) were lower than typical dairy farm fertility levels. The fertiliser was sprinkled evenly over the lysimeters/soil blocks and then washed into the soil by applying 2 L of water per lysimeter/soil block.

Experimental design and treatments

The experiment consisted of 12 treatments, each with five replicates (Table 24). There were three pasture types: (i) perennial ryegrass/white clover (PRG/WC), (ii) perennial ryegrass/white clover and plantain (PRG/WC/P) and (iii) Italian ryegrass/white clover and plantain (IRG/WC/P). All lysimeters/soil blocks received urine at a rate equivalent to 700 kg N ha^{-1}. Urine applications were split evenly between, February (late summer), March (early autumn), April (autumn) and May (late autumn) application dates. The treatments were arranged in the trench facility using a split plot design with urine application dates as the main-plot treatment factor and with pasture type being sub-plots.

Simulated grazing and trampling

Simulated grazing and trampling were performed the day before urine application. The lysimeters/soil blocks were trimmed to a residual mass equivalent of 1500 kg DM ha^{-1} using electric shears. The soil in all lysimeters/soil blocks was trampled (six 'stomps' each) using cow hoof simulation equipment designed to provide approximately 200 kPa, similar to the pressure exerted by an adult cow hoof (Di *et al*. 2001).

Urine collection and application

Urine was collected from lactating Holstein Friesian/Jersey cross cows on the Lincoln University Dairy Farm (LUDF). The cows were grazing standard PRG/WC diets, as this allowed pasture species composition and urine application date to be tested without the confounding factor of different chemical compositions of urine from different forages. Samples of the urine (50 mL) were taken and analysed for N concentration, using an Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany). Between collection and application urine was stored overnight in a refrigerator at 8ºC. Urine was applied at 2 L per lysimeter/soil block. Urine collection/application details are provided in Table 27.

Table 27. Details on urine collection, standardisation and application for each urine application date.

	February	**March**	**April**	**May**
Collection date	19 February 2019	19 March 2019	16 April 2019	14 May 2019
Application date	20 February 2019	20 March 2019	17 April 2019	15 May 2019
Herd size (Cattle)	300	300	300	220
Cattle diet	PRG/WC pasture (18 kg DM cow^{-1} day^{-1})	PRG/WC pasture (18 kg DM cow^{-1} day^{-1})	PRG/WC pasture (18 kg DM cow^{-1} day^{-1}) and PRG/WC silage (4.5 kg DM cow^{-1} day^{-1})	PRG/WC pasture (18 kg DM cow^{-1} day^{-1})
Urine volume collected (L)	64	75	75	70
Original urine concentration (g N L^{-1})	5.61	5.52	4.74	6.14
Urea added (g)	193.4	241.3	368.5	130.9
Final urine concentration (g N L^{-1})	6.96	6.96	6.73	6.69
Equivalent application rate (kg N ha^{-1})	709	709	686	681

Irrigation

Irrigation was applied to the lysimeters/soil blocks using a specially constructed small-scale pivot irrigation system to apply water at a rate of 5–10 mm per application. The irrigation scheduling from February-March simulated on-farm practices to replace evaporative losses. A total of 95 mm of

irrigation was applied during these months. A small amount of irrigation/simulated rainfall (16.8 mm) was also applied over the winter period, to ensure the treatments received a water input typical of the 75th percentile rainfall distribution for the location. Additional irrigation/simulated (30 mm) was applied on 3 September 2019 to create a leaching event, in order to ensure the NO_3^--N leaching breakthrough curves were completed.

Measurement methods

Nitrogen leaching losses

Leachate was collected from each lysimeter after each drainage event. The leachate drained through plastic tubing attached to an outlet nozzle at the bottom of the lysimeters, leading in to 10 L collection containers. Leachate volumes were measured and 50 mL sub-samples taken for chemical analysis. Samples were kept frozen at -20 °C until they were analysed for ammonium-N (NH_4^+-N) and NO_3^--N using a FOSS FIAstar 5000 twin channel analyser with SoFIA software version 2.00.

Pasture production and nitrogen uptake

Pasture on the lysimeters and soil blocks were harvested when plant development had reached the 2-3 leaf stage and yields were approximately 3000 kg DM ha^{-1}. The herbage was cut to a residual height of approximately 50 mm above ground level (1500 kg DM ha^{-1}). Dry matter production was determined gravimetrically after drying the herbage samples at 70°C for 72 hours. The dry herbage samples were then ground using a Retsch Ultra Centrifugal Mill ZM 200, with a 1 mm sieve, running at a speed of 18,000 rpm. The ground samples were stored in sealed 70 mL containers at room temperature in the dark, until analysis was conducted for total N content using an Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany). Nitrogen uptake was calculated by the product of DM yield and herbage N concentration.

Soil mineral nitrogen

Soil samples were taken from the soil blocks for analysis to elucidate the mechanisms involved in the transformation of soil mineral N. Samples were taken using a soil corer (100 mm depth, 75 mm diameter) on days 0, 1, 7, 14, 28, 56 and 112 after urine application. Holes remaining after core removal were immediately back-filled with a soil-sand mix and identified using plastic markers to prevent subsequent samplings occurring from that same position. The samples were stored at -80°C until analysis. Soil mineral N (NO_3^- and NH_4^+) concentration was determined on potassium chloride (KCl) extracts on a Lachat QuikChem 8500 Series 2 Flow Injection Analysis System (Lachat Instruments, Loveland, CO, USA), as described in Talbot *et al*. (2019). The average soil mineral N content for each treatment was calculated, over the period from day 1 to 112 after urine application, using the trapezoid rule to calculate the area under the curve (AUC) and dividing by 112.

The proportion of soil mineral N that is NO_3^--N was calculated by dividing the average soil NO_3^--N by the average soil mineral N and multiplying by 100.

Ammonia oxidising bacteria gene abundance

In high-N agricultural soils, as used in this study, nitrification is largely driven by soil ammonia oxidising bacteria (AOB), while ammonia oxidising archaea play a lesser role (Di *et al*. 2009b). Therefore, DNA extraction and soil AOB ammonia monooxygenase (amoA) gene abundance was measured using quantitative polymerase chain reaction (qPCR), following methodologies described in He *et al*. (2007). In brief, DNA was extracted from the soil using a NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany) as per the manufacturer's instructions. AOB amoA gene abundance was measured using real time qPCR on a Rotor-Gene™ 6000 (Corbett Research, Australia) using the parameters as described in (Di *et al*. 2009b). Data were then analysed using the Rotor-Gene™ series software 1.7.

Statistical analysis

The data sets were subject to analysis of variance (ANOVA) (Split-Plot Design) using Genstat (18th edition, VSN International Ltd.). The AOB *amoA* gene abundance data set was log-transformed to ensure homogeneity of residual errors.

Results

Climate conditions and water inputs

Over the experimental period (20 February 2019 to 16 September 2019), the average daily air temperature ranged from a high of 23.4 °C in March 2019 to a low of 1.0 °C in June 2019 (Figure 43a). Daily average soil temperature (100 mm depth) ranged from a high of 24.3 °C in February 2019 to a low of 4.2 °C in June 2019 (Figure 43a). Water inputs for the experimental period totalled 621 mm, comprising of 439 mm of rainfall, 142 mm of irrigation and 40 mm of water inputs from the urine application (Figure 43b).

Leaching losses

Nitrate concentration in leachate

The NO_3^--N concentration peaks in the complete breakthrough curves for PRG/WC/P (139-370 mg NO_3^--N L^{-1}) and IRG/WC/P (141-395 mg NO_3^--N L^{-1}) were lower than the PRG/WC (174-438 mg NO_3^--N L^{-1}) peaks, for all four separate urine application months (Figure 44). The lowest NO_3^--N concentration peaks for each pasture type occurred from the May urine application, while the highest NO_3^--N concentration peaks were occurred from the March or April urine applications.

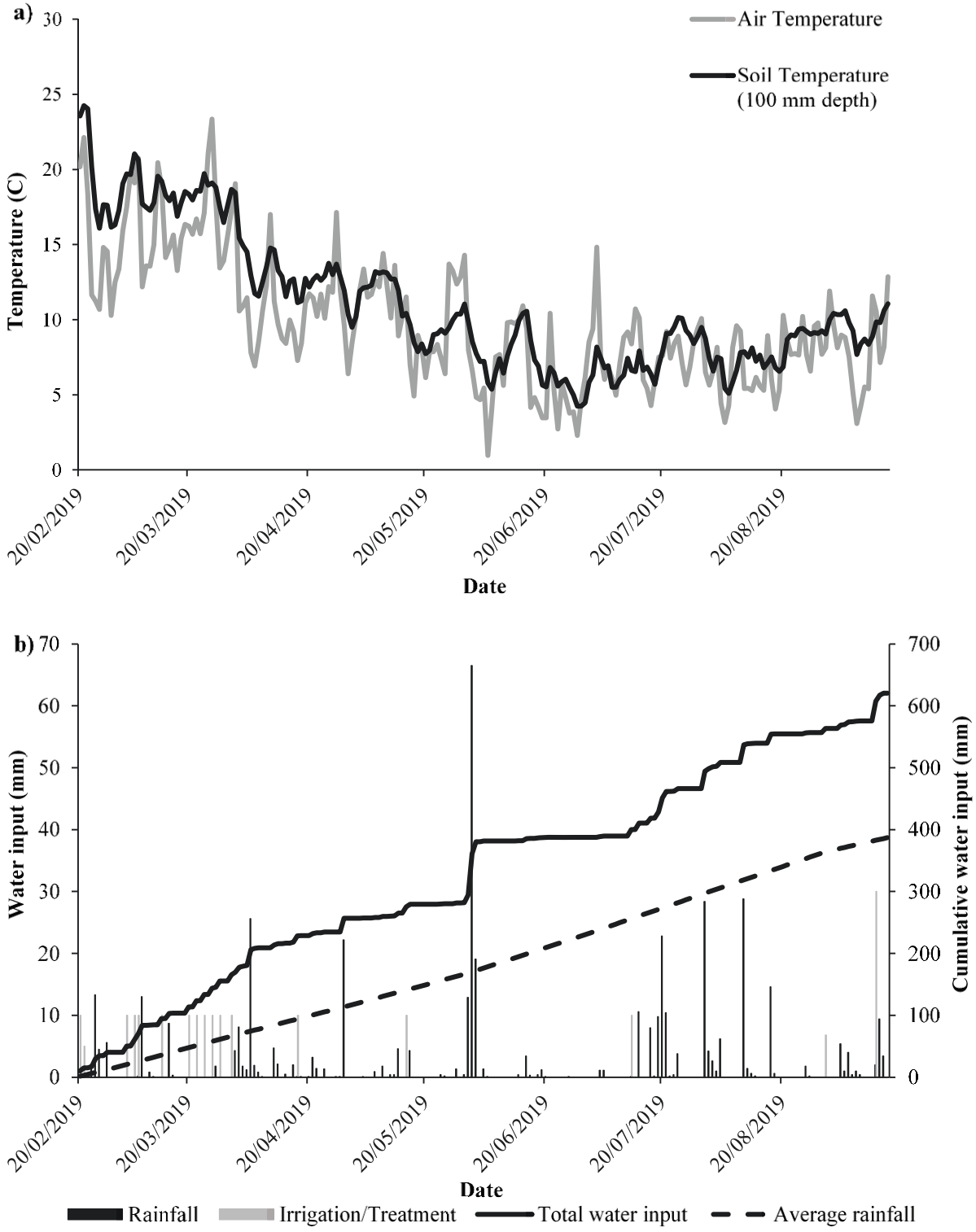


Figure 43. a) Average daily air temperature and soil temperature (at 100 mm) and b) daily rainfall, irrigation/treatment water inputs, cumulative water inputs over the experimental period (20 February 2019-16 September 2019), and long-term average rainfall data (1971-2000).

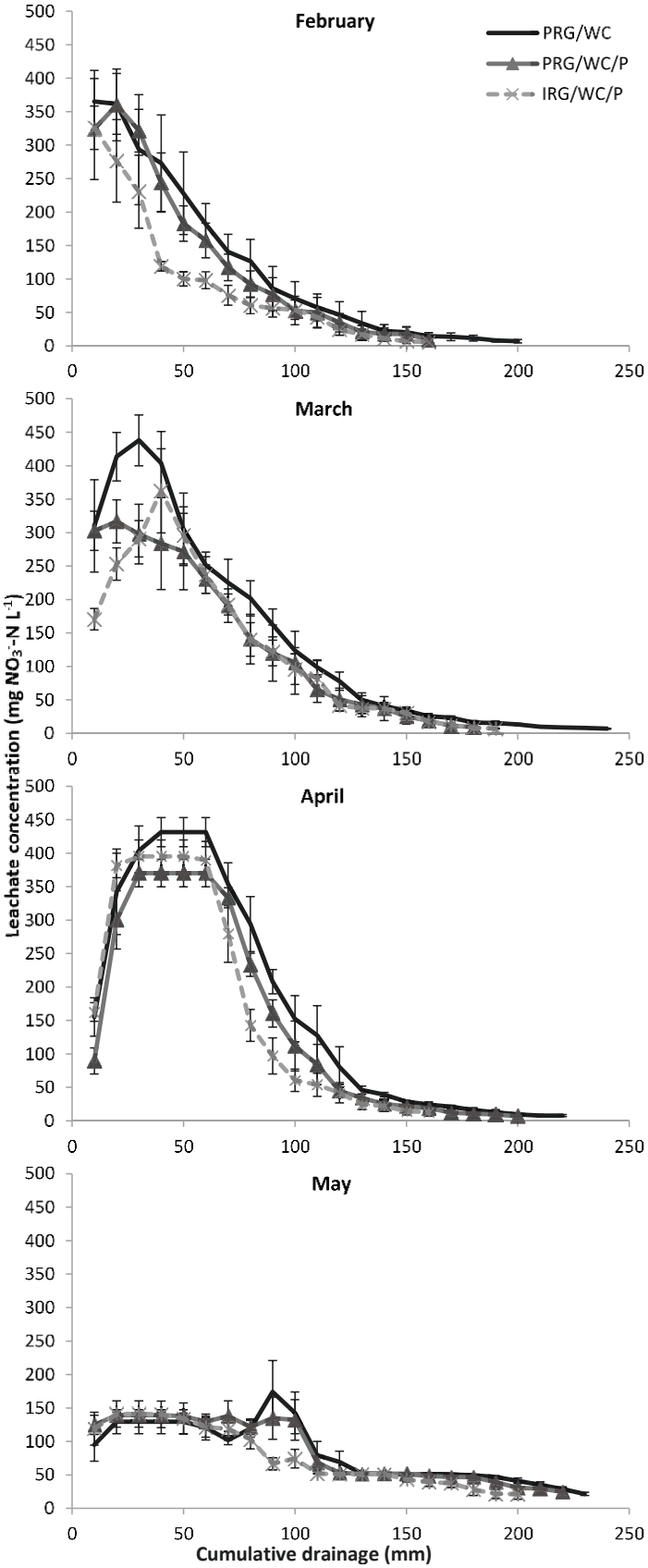


Figure 44. Mean nitrate concentration in leachate plotted against cumulative drainage (mm) over the sampling periods, under the different pastures; perennial ryegrass/white clover (PRG/WC), perennial ryegrass/white clover/plantain (PRG/WC/P) and Italian ryegrass/white clover/plantain (IRG/WC/P).

Nitrate leaching and drainage losses

Comparison of the main effects of each pasture type, detected a significant ($P < 0.05$) reduction in total NO_3^--N leached under the PRG/WC/P (14% reduction) and IRG/WC/P (24% reduction) compared with the PRG/WC (Table 28). The IRG/WC/P leached 34%, 25%, 19% and 19% less NO_3^--N than the PRG/WC in February, March, April and May, respectively (Figure 45); however, the difference in May was not statistically significant. The PRG/WC/P leached 9%, 22%, 17% and 2% less NO_3^--N than the PRG/WC in February, March, April and May, respectively. However, only the 22% reduction in March was statistically significant.

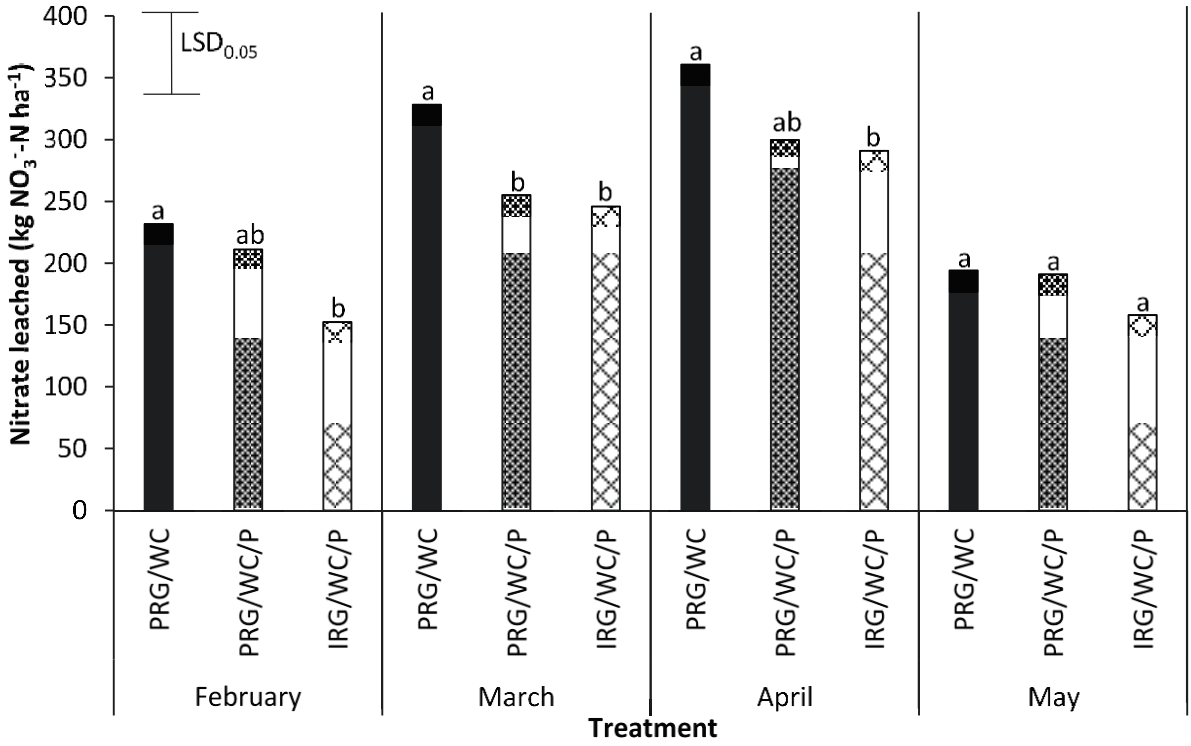


Figure 45. Total nitrate leached over the sampling periods, under each treatment. Least significant difference (LSD), for comparisons within a urine application date, is at the 5% level (n = 5). LSD = 61.6. Bars, within the same urine application date, with a letter in common are not significantly different at the 5% level. Perennial ryegrass/white clover (PRG/WC). Perennial ryegrass/white clover/plantain (PRG/WC/P). Italian ryegrass/white clover/plantain (IRG/WC/P).

There was a significant ($P < 0.05$) main treatment effect of urine timing on the total amount of NO_3^--N leached. April urine applications had the highest average NO_3^--N leaching losses; followed by March, losses for February and May, which were not significantly different (Table 28).

Table 28. The effect each treatment had on total nitrate-nitrogen (NO_3^--N), ammonium-N (NH_4^+-N) and mineral N leached, and total drainage over the sampling periods, plus the main effect of pasture type and urine application month. Main effect values with a letter in common are not significantly different at the 5% level. Perennial ryegrass/white clover (PRG/WC). Perennial ryegrass/white clover/plantain (PRG/WC/P). Italian ryegrass/white clover/plantain (IRG/WC/P).

Urine application date (measurement period)	Pasture type	Nitrate leached (kg NO_3^--N ha^{-1})	Ammonium leached (kg NH_4^+-N ha^{-1})	Mineral N leached (kg N ha^{-1})	Drainage volume (mm)
February (209 days)	**PRG/WC**	231.6	3.3	234.9	232.4
	PRG/WC/P	211.0	3.4	214.4	234.8
	IRG/WC/P	152.3	4.5	156.9	225.8
March (181 days)	**PRG/WC**	328.2	28.0	356.2	268.7
	PRG/WC/P	255.2	17.0	272.2	232.5
	IRG/WC/P	245.8	31.4	277.2	247.6
April (153 days)	**PRG/WC**	360.6	12.7	373.3	238.1
	PRG/WC/P	299.7	17.6	317.3	231.1
	IRG/WC/P	290.9	18.3	309.2	204.6
May (125 days)	**PRG/WC**	194.0	58.7	252.8	256.9
	PRG/WC/P	190.9	33.0	223.9	241.8
	IRG/WC/P	158.1	40.2	198.2	215.1
LSD (5%) (*n*=5):					
Comparisons within a urine application date		61.6	22.2	68.2	42.1
Other comparisons		61.7	19.0	62.8	39.9
Main effect of pasture type	PRG/WC	278.6^{a}	25.7^{a}	304.3^{a}	249.0^{a}
	PRG/WC/P	239.2^{b}	17.8^{a}	257.0^{b}	235.1^{ab}
	IRG/WC/P	211.8^{b}	23.6^{a}	235.4^{b}	223.3^{b}
LSD (5%)		30.8	9.5	31.4	19.9
Main effect of urine application month	February	198.3^{c}	3.8^{c}	202.1^{b}	231.0^{a}
	March	276.4^{b}	25.5^{b}	301.8^{a}	249.6^{a}
	April	317.1^{a}	16.2^{bc}	333.2^{a}	224.6^{a}
	May	181.0^{c}	44.0^{a}	225.0^{b}	237.9^{a}
LSD (5%)		39.2	17.1	48.9	29.3

Total leaching losses

There was no significant main effect among the pasture types for the total amount of NH_4^+-N leached; however, urine application date had a significant ($P < 0.05$) effect on total amount of NH_4^+-N leached (Table 28). The May urine application had significantly higher average NH_4^+-N leaching loss than the other months; followed by March and April, which were not significantly different. February applications resulted in the lowest amount of NH_4^+-N leached.

The IRG/WC/P had significantly ($P < 0.05$) lower average drainage loss than the PRG/WC, while the drainage volume for the PRG/WC/P was not significantly different to the other two pasture types (Table 28). There was no significant difference in average drainage between the four urine timing treatments.

Herbage yield and nitrogen uptake

Yield

The pasture type had a significant ($P < 0.05$) main effect on total dry matter yield (Table 29) The IRG/WC/P had significantly ($P < 0.05$) higher total dry matter yield than the PRG/WC. The PRG/WC/P also had marginally higher total dry matter yield than the PRG/WC, however, this difference was not significant. Pasture type had a significant main effect on the dry matter yield over the mid-autumn to mid-winter period (Table 29). The IRG/WC/P had significantly higher dry matter yield than the PRG/WC and the PRG/WC/P, over the mid-autumn to mid-winter period.

Plant nitrogen uptake

Pasture type had no significant effect on total plant N uptake over the whole experiment (Table 29). However, there was a significant effect of pasture type on plant N uptake over the mid-autumn to mid-winter period (Table 29). The IRG/WC/P had significantly higher plant N uptake than the PRG/WC and the PRG/WC/P.

Table 29. Treatment effects on herbage yield (kg DM ha^{-1}) and plant uptake (kg N ha^{-1}), plus the main effect of pasture type and urine application month. Main effect values with a letter in common are not significantly different at the 5% level. Perennial ryegrass/white clover (PRG/WC). Perennial ryegrass/white clover/plantain (PRG/WC/P). Italian ryegrass/white clover/plantain (IRG/WC/P).

		Total over experimental period (20 February 2019 – 16 September 2019)		Mid Autumn – Mid winter period (17 April 2019 – 17 July 2019)	
Urine application date (Total measurement period: Mid Autumn –Mid winter period measurement period)	**Pasture type**	**Herbage yield (kg DM ha^{-1})**	**Plant N uptake (kg N ha^{-1})**	**Herbage yield (kg DM ha^{-1})**	**Plant N uptake (kg N ha^{-1})**
February (209 days: 92 days)	**PRG/WC**	8544	357.8	2670	106.1
	PRG/WC/P	7773	313.7	2014	80.5
	IRG/WC/P	8500	320.8	2675	96.4
March (181 days: 92 days)	**PRG/WC**	5692	246.3	1922	89.3
	PRG/WC/P	6453	267.7	2552	110.7
	IRG/WC/P	6121	231.1	2660	105.8
April (153 days: 92 days)	**PRG/WC**	3950	168.0	2071	97.3
	PRG/WC/P	4222	172.6	2238	105.2
	IRG/WC/P	4686	183.1	2580	120.5
May (125 days: 64 days)	**PRG/WC**	2573	99.8	657	33.2
	PRG/WC/P	2884	113.3	796	38.6
	IRG/WC/P	3969	134.5	1280	64.9
LSD (5%) (*n*=5):					
Comparisons within a urine application date		1036	42.3	604	24.1
Other comparisons		951	38.2	513	18.4
Main effect of pasture type	PRG/WC	5190^{b}	218.0^{a}	1830^{b}	81.5^{b}
	PRG/WC/P.	5333^{ab}	216.8^{a}	1900^{b}	83.8^{b}
	IRG/WC/P	5819^{a}	217.4^{a}	2299^{a}	96.9^{a}
LSD (5%)		475	19.1	256	9.2
Main effect of urine application date	February	8272^{a}	330.8^{a}	2453^{a}	94.3^{a}
	March	6089^{b}	248.4^{b}	2378^{a}	102.0^{a}
	April	4286^{c}	174.6^{c}	2296^{a}	107.7^{a}
	May	3142^{d}	115.9^{d}	911^{b}	45.6^{b}
LSD (5%)		743.9	30.9	468	19.9

Soils

Mineral N

Soil mineral N (NO_3^--N + NH_4^+-N) was highest in all treatments immediately following urine application (Figure 46), and thereafter, a continual decrease in soil mineral N was observed, under all treatments. The pasture type had a significant ($P < 0.05$) effect on average soil mineral N, with IRG/WC/P having significantly lower average soil mineral N (104 kg N ha^{-1}) than RG/WC (124 kg N ha^{-1}). This effect was largely due to the lower soil mineral N under IRG/WC/P in the colder, April and May urine application dates (Figure 46). The PRG/WC/P average soil mineral N (110.9 kg N ha^{-1}) was not significantly different from the two other pasture types. The IRG/WC/P and PRG/WC/P had slightly lower average soil NO_3^--N (69.6 kg NO_3^--N ha^{-1} and 70.0 kg NO_3^--N ha^{-1}, respectively) than the PRG/WC (78.8 kg NO_3^--N ha^{-1}), however, this difference was not significant. The IRG/WC/P had a significantly ($P < 0.05$) lower average soil mineral NH_4^+-N (34.5 kg NH_4^+-N ha^{-1}) than the PRG/WC (44.8 kg NO_3^--N ha^{-1}); while the PRG/WC/P average soil mineral NO_3^--N (40.8 kg NO_3^--N ha^{-1}) was not significantly different from the other two pasture types.

The average proportion of soil mineral N present as NO_3^--N 14 days after urine application was calculated for each urine application. The soil mineral N was comprised of mainly NO_3^--N in the February (95%), March (97%) and April (62%) urine applications, compared with the May (40%) urine application.

AOB gene abundance

The effect of urine application date and pasture type on AOB *amoA* gene abundance over the first 112 days after urine application is shown in Figure 47. Using the AUC for the first 112 days after urine application and log transforming the data set, urine application date ($P = 0.423$) and pasture type ($P = 0.680$) were shown to have no significant effect on AOB *amoA* gene abundance.

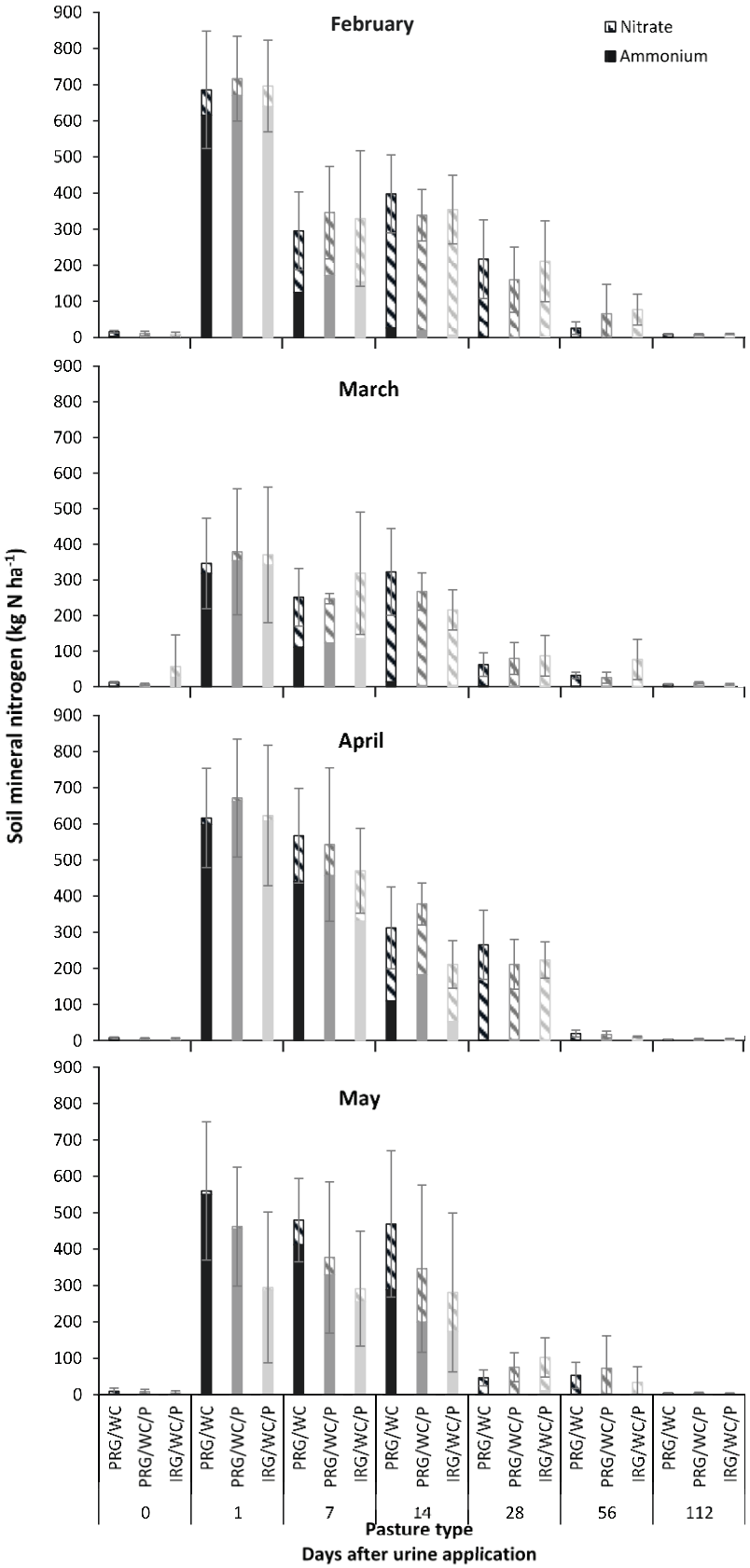


Figure 46. Treatment effects on the soil mineral nitrogen (NO_3^--N + NH_4^+-N) over the sampling periods. Soil samples were taken from 0-100 mm depth. Error bars are standard error of the mean for total mineral nitrogen (*n* = 5). Perennial ryegrass/white clover (PRG/WC). Perennial ryegrass/white clover/plantain (PRG/WC/P). Italian ryegrass/white clover/plantain (IRG/WC/P).

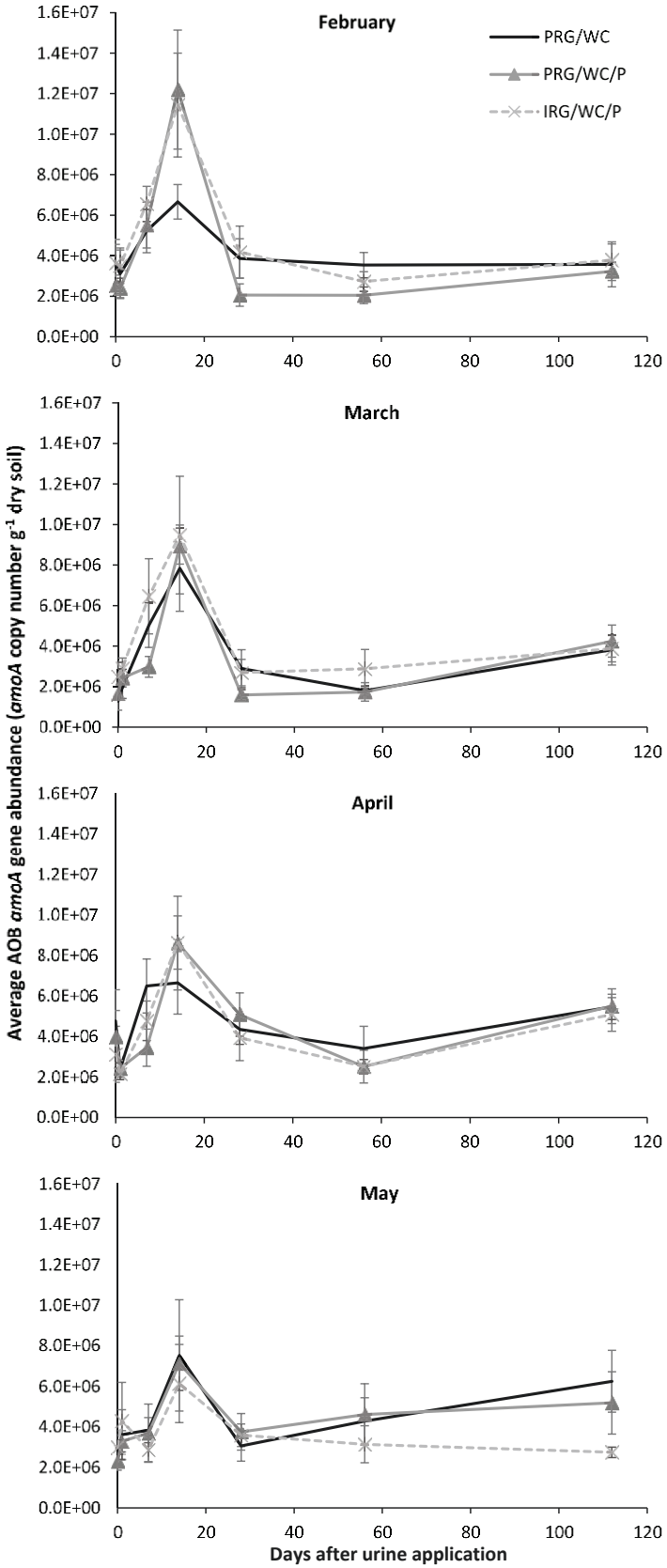


Figure 47. Treatment effects on soil AOB *amoA* gene copies over the first 112 days after urine application. Soil samples were taken from 0-100 mm depth. Error bars are standard error of the mean (*n* = 5). Perennial ryegrass/white clover (PRG/WC). Perennial ryegrass/white clover/plantain (PRG/WC/P). Italian ryegrass/white clover/plantain (IRG/WC/P)

Discussion

Pasture effect

IRG/WC/P vs PRG/WC

There was a significant difference in mid-autumn to mid-winter herbage yield and plant N uptake, between IRG/WC/P (2299 kg DM ha^{-1} and 97 kg N ha^{-1}) and PRG/WC (1830 kg DM ha^{-1} and 82 kg N ha^{-1}). These results are consistent with literature that suggests IRG has greater winter growth than PRG (Malcolm *et al*. 2014; Woods *et al*. 2016; Woods *et al*. 2018). The increase in mid-autumn to mid-winter plant N uptake resulted in the significantly lower average soil mineral N content under the IRG/WC/P (104 kg N ha^{-1}) compared with the PRG/WC (124 kg N ha^{-1}) (Figure 46). The difference in soil mineral N between the pasture types was greater for the urine application treatments in the colder months (April and May) (Figure 46). This is consistent with the effects of higher mid-autumn to mid-winter plant N uptake by IRG.

The IRG/WC/P treatment leached on average 24% less NO_3^--N than PRG/WC for each urine application date (Table 28). This reduction in NO_3^--N leaching under the IRG/WC/P is attributable to a combination of lower NO_3^--N concentration in the leachate (Figure 44) and a lower drainage volume (Table 28). The higher plant N uptake, under the IRG/WC/P, led to a decrease in the soil mineral N content and subsequently a reduction in the amount of NO_3^--N available to be leached. Therefore the lower NO_3^--N concentration peaks, under the IRG/WC/P, are attributed to the higher plant N uptake (Table 29). The lower drainage losses in the IRG/WC/P treatment (Table 28) is also attributed to increased herbage yield, because of increased rates of transpiration depleting the soil moisture content. These reductions in NO_3^--N leaching under the IRG/WC/P are consistent with earlier studies that showed that a reduction in NO_3^--N leaching of 24-54% is expected when IRG is incorporated into pastures, compared with traditional PRG (Malcolm *et al*. 2014; Woods *et al*. 2016; Maxwell *et al*. 2018). This reduction in NO_3^--N leaching losses shows the importance of winter growth and the potential of IRG in reducing NO_3^--N losses from farmland and highlights the benefits of breeding pasture species that are more winter active.

PRG/WC/P vs PRG/WC

Dry matter yield and N uptake by PRG/WC/P showed similar trends to IRG/WC/P when compared with PRG/WC, however, these differences were notably smaller.

There was a significant main effect of pasture type on NO_3^--N leaching losses (Table 28), with a 14% reduction in NO_3^--N leached beneath PRG/WC/P compared with the PRG/WC ($P < 0.05$). This significant difference in the amount of NO_3^--N leached from PRG/WC/P compared with PRG/WC resulted from

the March urine application treatment, where a 22% reduction was observed. This reduction in NO_3^--N leaching is attributed to the marginally higher mid-autumn to mid-winter dry matter yield and plant N uptake under the PRG/WC/P, compared with the PRG/WC.

The reduction in NO_3^--N leaching under the PRG/WC/P treatment compared with PRG/WC in our study (14%) is notably lower than that reported in the literature, where it has been shown that plantain can reduce NO_3^--N leaching losses by up to 82% (Carlton *et al*. 2018; Welten *et al*. 2019). This lower reduction may be due to the shallow stony soil type in the current study (compared to the deeper Horotiu silt loam and Paparua fine sandy loam soils, used by Welten *et al*. (2019) and Carlton *et al*. (2018), respectively. The free draining shallow stony soil allows rapid N leaching to occur, and therefore there may have been insufficient time for any potential direct effect of plantain roots on N transformations. In addition, previous studies used cow urine collected from cows on a plantain diet and applied at lower urine-N rates, suggesting that reductions in N leaching by plantain pastures might be more strongly influenced by urine composition and urine-N application rate than any direct associations of plantain root systems on soil N processes.

Timing of urine deposition

The nitrification rate appears to have been higher following urine applications in warmer months, compared with applications during the cooler months. This is shown in Figure 46, where 14 days after urine application the soil mineral N is composed mainly of NO_3^--N in the February (late summer) (95.4%). For subsequent months of application, the proportions of nitrate on day 14 progressively declined, reaching a minimum of 39.6% for May (late autumn) urine applications. The higher nitrification rates in warmer conditions are consistent with literature (Stark 1996; Sahrawat 2008). There was also high plant growth/high plant N uptake in the warmer conditions (Table 29); the earlier the urine application, the more time there was available for plants to uptake the urine-N, before the majority of the leaching occurred in June-August (winter). The nitrification rate and plant growth/plant N uptake were important factors in the N leaching losses.

The lowest NO_3^--N leaching losses were found from the May (181 kg NO_3^--N ha^{-1}) and February (198 kg NO_3^--N ha^{-1}) urine applications. The low NO_3^--N losses in May were attributed to the low nitrification rate in the colder conditions, reducing the amount of soil NO_3^--N available to be leached over the winter period; this allowed the N to be held in the soil profile, in less mobile forms (e.g. NH_4^+-N) and subsequently be up taken by plants in warmer conditions in spring). The low NO_3^--N leaching losses following the February urine application were attributed to the higher plant N uptake in the warmer conditions, reducing soil mineral N before significant leaching occurred over the June-August period. The March and April urine applications had significantly higher NO_3^--N leaching losses

(276 kg NO_3^--N ha^{-1} and 317 kg NO_3^--N ha^{-1}, respectively). This was attributed to the high nitrification rate in March and April, compared to May, and lower plant N uptake, compared with February.

In previous studies high NO_3^--N leaching losses from cow urine patches have been observed under pasture (60–416 NO_3^--N ha^{-1}), when urine (700-1000 kg N ha^{-1}) has been applied in April or May (Cameron *et al*. 2007; Malcolm *et al*. 2014; Woods *et al*. 2016; Carlton *et al*. 2018; Maxwell *et al*. 2018; Talbot *et al*. 2019). The amounts of NO_3^--N leaching losses recorded in the current study are high compared with limited literature values (40–126 NO_3^--N ha^{-1}) when urine (622–700 kg N ha^{-1}) was applied to pasture in February or March (Carlton *et al*. 2018; Woods *et al*. 2018; Welten *et al*. 2019). However, these studies were performed on deep, stone-free soils, suggesting that NO_3^--N leaching losses may be higher than expected when urine is deposited onto shallow stony soils, during warmer months (February-March).

Conclusions

The main conclusion of the research was there was a significant reduction in the total amount of NO_3^--N leached under the PRG/WC/P (14% reduction) and IRG/WC/P (24% reduction) compared with the PRG/WC. The reasons for these reductions were an increase in plant N uptake, which decreased the soil mineral N content and subsequently reduced the amount of N available to be leached; and an increase in herbage yield which increased transpiration, thus reducing drainage volume.

Chapter 7

Key findings and conclusions

7.1 Lysimeter experiment 1 - key findings

7.1.1 Trial 1 (Talbot *et al*. 2019)

-Applying readily available C to soil can significantly reduce N leaching losses without causing an increase in N_2O emissions. This was attributed to the added C increasing immobilisation of N in the soil.

-The PRG/WC pasture leached 58% less N than the lucerne crop. This was attributed to the higher winter plant growth/N uptake of the PRG/WC pasture, reducing the amount of mineral N available for leaching.

7.1.2 Trial 2 (Talbot *et al*. 2020)

-Urine from cows fed on a diet of FB and applied onto a PRG/WC pasture leached 64% less NO_3^--N than urine from cows fed on a diet of PRG/WC and applied onto a PRG/WC pasture, even when applied at the same urine N loading rate. The soil under the FB urine patches had significantly lower AOB *amoA* gene abundance and lower amount of soil NO_3^--N, suggesting that the FB urine had a biological nitrification inhibitor (BNI) effect.

-The PRG/WC pasture leached 65-84% less N than the amount leached from bare fallow FB soil. This was attributed to the higher winter plant growth/N uptake of the PRG/WC pasture, reducing the amount of mineral N available to be leached.

7.2 Lysimeter experiment 2 - key findings

- Lowering the urine loading rate by 28%, from 700 kg N ha^{-1} to 500 kg N ha^{-1}, produced a 38% reduction in N_2O emissions and a 39% reduction in total N leaching losses. This demonstrates that reducing urine-N concentration or amount in each urine patch could be an effective way of reducing N losses from grazing systems.

7.3 Lysimeter experiment 3 - key findings

-Urine applied to IRG/WC/P had significantly lower N leaching losses compared with traditional PRG/WC (24% lower). This was attributed to the significantly higher late autumn - mid winter plant growth of the IRG/WC/P.

-Urine applied to PRG/WC/P had significantly lower N leaching losses compared with traditional PRG/WC (14% lower). This was attributed to the slightly higher late autumn - mid winter plant growth of the PRG/WC/P. No BNI effect was seen under the PRG/WC/P compared with PRG/WC.

-These significant differences in losses highlight the potential of using more winter active plant species or cultivars in pastures to reduce N leaching losses.

-Nitrate leaching losses may be higher than expected when urine is deposited onto shallow stony soils, during warmer months (February-March).

7.4 Final conclusions

This research programme through manipulating different C inputs, has highlighted the importance of winter growth and the manipulation of cattle diet in reducing N losses.

-Increasing plant N uptake over cooler months is potentially an economically viable and effective way of reducing farm N leaching losses. This can be achieved by sowing more winter active crops/pastures, reducing time spent by cattle on bare fallow soil and/or through the use of effective catch crops.

-Manipulating cattle diet through low N feed and subsequently reducing the urine N loading rate is potentially an economically viable and effective way of reducing farm N leaching losses; especially if a low N feed can create a BNI effect in the cattle urine, such as detected with fodder beet.

7.5 Suggestions for future research

-More research is needed to help develop effective and economic strategies that enable farmers to reduce cattle urine-N loading rates.

-Identifying plants that sequester C or release important C based compounds (e.g. biological nitrification inhibitors) could be a viable area of research that could result in the tightening of the N cycling in farm systems.

-Further research is needed to explore the potential to manipulate urine-C composition through diet manipulation, to reduce N leaching losses from urine patches.

- Further research is needed to identify the compound(s) responsible for the BNI effect observed under the beet urine treatment

-Breeding pasture species that are more winter active.

Appendix A

Lysimeter experiment 1 treatment layout

N

Treatment no.	Lysimeter
4	**1 (1)**
7	**2 (1)**
8	**3 (1)**
3	**4 (1)**
2	**5 (1)**
1	**6 (1)**
6	**7 (1)**
5	**8 (1)**
6	**9 (2)**
3	**10 (2)**
5	**11 (2)**
2	**12 (2)**
7	**13 (2)**
4	**14 (2)**
8	**15 (2)**
1	**16 (2)**
8	**17 (3)**
1	**18 (3)**
2	**19 (3)**
5	**20 (3)**

Trench

Lysimeter	Treatment no.
40 (5)	7
39 (5)	4
38 (5)	3
37 (5)	6
36 (5)	8
35 (5)	1
34 (5)	2
33 (5)	5
32 (4)	1
31 (4)	7
30 (4)	5
29 (4)	2
28 (4)	8
27 (4)	6
26 (4)	3
25 (4)	4
24 (3)	6
23 (3)	4
22 (3)	3
21 (3)	7

(*) is statistical block

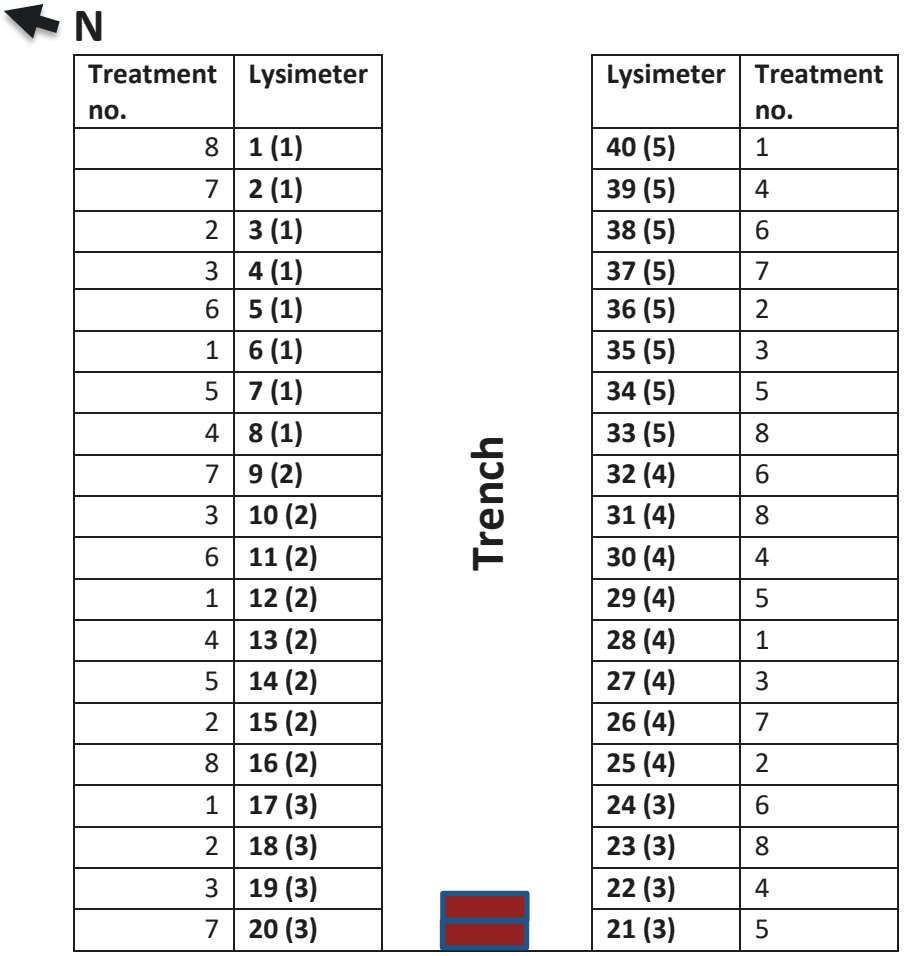
Bethels Road

Table A-1. Treatments applied to the lysimeters and soil blocks in lysimeter experiment 1. Perennial ryegrass/white clover pasture (PRG/WC). Fodder beet (FB). Carbon (C).

Treatment no.	Crop type	Urine type	Urine rate (kg N ha^{-1})	Carbon input	Repetitions
1	FB	FB	700	-	5
2	FB	PRG/WC	700	-	5
3	PRG/WC	FB	700	-	5
4	PRG/WC	PRG/WC	700	-	5
5	PRG/WC	PRG/WC	700	C rate 1 (12 t sucrose ha^{-1})	5
6	PRG/WC	PRG/WC	700	C rate 2 (24 t sucrose ha^{-1})	5
7	Lucerne	PRG/WC	700	-	5
8	Lucerne	PRG/WC	700	C rate 1 (12 t sucrose ha^{-1})	5

Appendix B

Lysimeter experiment 2 treatment layout



Treatment no.	Lysimeter
8	**1 (1)**
7	**2 (1)**
2	**3 (1)**
3	**4 (1)**
6	**5 (1)**
1	**6 (1)**
5	**7 (1)**
4	**8 (1)**
7	**9 (2)**
3	**10 (2)**
6	**11 (2)**
1	**12 (2)**
4	**13 (2)**
5	**14 (2)**
2	**15 (2)**
8	**16 (2)**
1	**17 (3)**
2	**18 (3)**
3	**19 (3)**
7	**20 (3)**

Lysimeter	Treatment no.
40 (5)	1
39 (5)	4
38 (5)	6
37 (5)	7
36 (5)	2
35 (5)	3
34 (5)	5
33 (5)	8
32 (4)	6
31 (4)	8
30 (4)	4
29 (4)	5
28 (4)	1
27 (4)	3
26 (4)	7
25 (4)	2
24 (3)	6
23 (3)	8
22 (3)	4
21 (3)	5

(*) is statistical block

Table B-1. Treatments applied to the lysimeters and soil blocks in lysimeter experiment 2. Perennial ryegrass/white clover (PRG/WC). Perennial ryegrass/white clover/plantain (PRG/WC/P).

Treatment no.	Pasture type	Urine type	Urine rate (kg N ha^{-1})	Replicates
1	PRG/WC	PRG/WC	500	5
2	PRG/WC	PRG/WC	700	5
3	PRG/WC	PRG/WC/P	500	5
4	PRG/WC	PRG/WC/P	700	5
5	PRG/WC/P	PRG/WC	500	5
6	PRG/WC/P	PRG/WC	700	5
7	PRG/WC/P	PRG/WC/P	500	5
8	PRG/WC/P	PRG/WC/P	700	5

N

Lysimeter experiment 3 treatment layout

Treatment no.	Lysimeter		Lysimeter	Treatment no.
8	**1 (1)**		**60 (5)**	7
7	**2 (1)**		**59 (5)**	9
9	**3 (1)**		**58 (5)**	8
3	**4 (1)**		**57 (5)**	10
1	**5 (1)**		**56 (5)**	12
2	**6 (1)**		**55 (5)**	11
4	**7 (1)**		**54 (5)**	5
6	**8 (1)**		**53 (5)**	6
5	**9 (1)**		**52 (5)**	4
10	**10 (1)**		**51 (5)**	1
12	**11 (1)**		**50 (5)**	2
11	**12 (1)**		**49 (5)**	3
9	**13 (2)**	**Trench**	**48 (4)**	7
7	**14 (2)**		**47 (4)**	9
8	**15 (2)**		**46 (4)**	8
2	**16 (2)**		**45 (4)**	1
3	**17 (2)**		**44 (4)**	2
1	**18 (2)**		**43 (4)**	3
5	**19 (2)**		**42 (4)**	12
6	**20 (2)**		**41 (4)**	11
4	**21 (2)**		**40 (4)**	10
11	**22 (2)**		**39 (4)**	6
10	**23 (2)**		**38 (4)**	5
12	**24 (2)**		**37 (4)**	4
6	**25 (3)**		**36 (3)**	1
5	**26 (3)**		**35 (3)**	2
4	**27 (3)**		**34 (3)**	3
8	**28 (3)**		**33 (3)**	11
7	**29 (3)**		**32 (3)**	12
9	**30 (3)**		**31 (3)**	10

(*) is statistical block

Table C-1. Treatments applied to the lysimeters and soil blocks in lysimeter experiment 3. Perennial ryegrass/white clover (PRG/WC). Perennial ryegrass/white clover/plantain (PRG/WC/P). Italian ryegrass/white clover/plantain (IRG/WC/P).

Treatment no.	Pasture type	Urine application date	Urine rate (kg N ha^{-1})	Replicates
1	PRG/WC	February	700	5
2	PRG/WC/P	February	700	5
3	IRG/WC/P	February	700	5
4	PRG/WC	March	700	5
5	PRG/WC/P	March	700	5
6	IRG/WC/P	March	700	5
7	PRG/WC	April	700	5
8	PRG/WC/P	April	700	5
9	IRG/WC/P	April	700	5
10	PRG/WC	May	700	5
11	PRG/WC/P	May	700	5
12	IRG/WC/P	May	700	5

Appendix D

Soil map of Ashley Dene Research Development Station

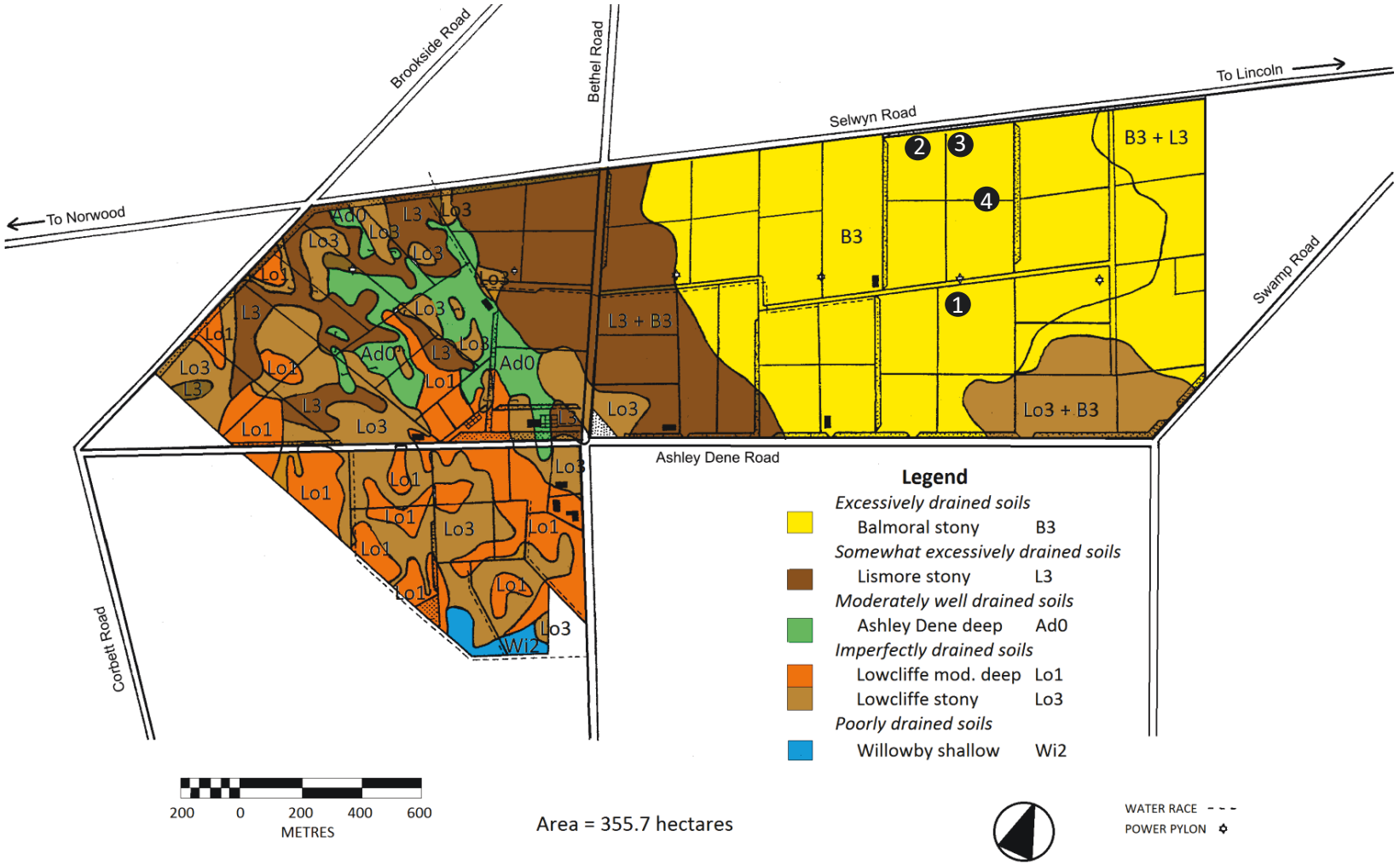


Figure D-1. Soil map of Ashley Dene Research and Development Station, showing location of lysimeter collection sites: Lucerne = 1. Fodder beet = 2. Perennial ryegrass/white clover =3. Plantain/white clover = 4. Adapted from Webb and Bennett (1986).

Appendix E

Lysimeter collection sites

Soil profile	Collection site	Paddock history	Lysimeters collected
	Lucerne (43°38′51.8″S 172° 21′01.0″E)	This paddock was under sheep grazed cocksfoot pasture then converted to lucerne in 2015. The lucerne cultivar was Stamina 5, sown at a rate of 10 kg ha^{-1}.	In summer 2016/17, ten lysimeters were collected, for lysimeter experiment 1.

	Fodder beet (43°38'38.1"S 172°20'42.0"E).	The paddock was under PRG/WC pasture then it was converted to winter grazed kale for winter 2015. In autumn 2015 the paddock was converted to fodder beet 'Jamon', sown at a rate of 100,000 seeds ha^{-1}. The paddock was winter grazed in 2016 and resown in fodder beet in autumn 2016.	In summer 2016/17, ten lysimeters were collected, for lysimeter experiment 1.
	Perennial ryegrass/white clover (PRG/WC) (43°38′37.7″S 172°20′38.8″E)	The paddock was under winter grazed FB for winter 2013, then it was converted to PRG/WC pasture in autumn 2013. The PRG cultivar was 'Prospect' with AR37 endophyte, sown at a rate of 18 kg ha^{-1}. The WC cultivar was 'Legacy', sown at 5 kg ha^{-1}.	In summer 2016/17, twenty lysimeters were collected, for lysimeter experiment 1. In summer 2017/18, twenty lysimeters were collected, for lysimeter experiment 2.

	Plantain/white clover (43°38'42.9"S 172°20'51.0"E)	This paddock was sown in plantain and WC in autumn 2016. The plantain cultivar was 'Tonic', sown at a rate of 9 kg ha^{-1}. The WC cultivar was 'Legacy', sown at a rate of 6 kg ha^{-1}.	In summer 2017/18, twenty lysimeters were collected, for lysimeter experiment 2. In summer 2018/19, sixty lysimeters were collected, for lysimeter experiment 3.

Appendix F

Supplementary material for Talbot *et al.* (2020)

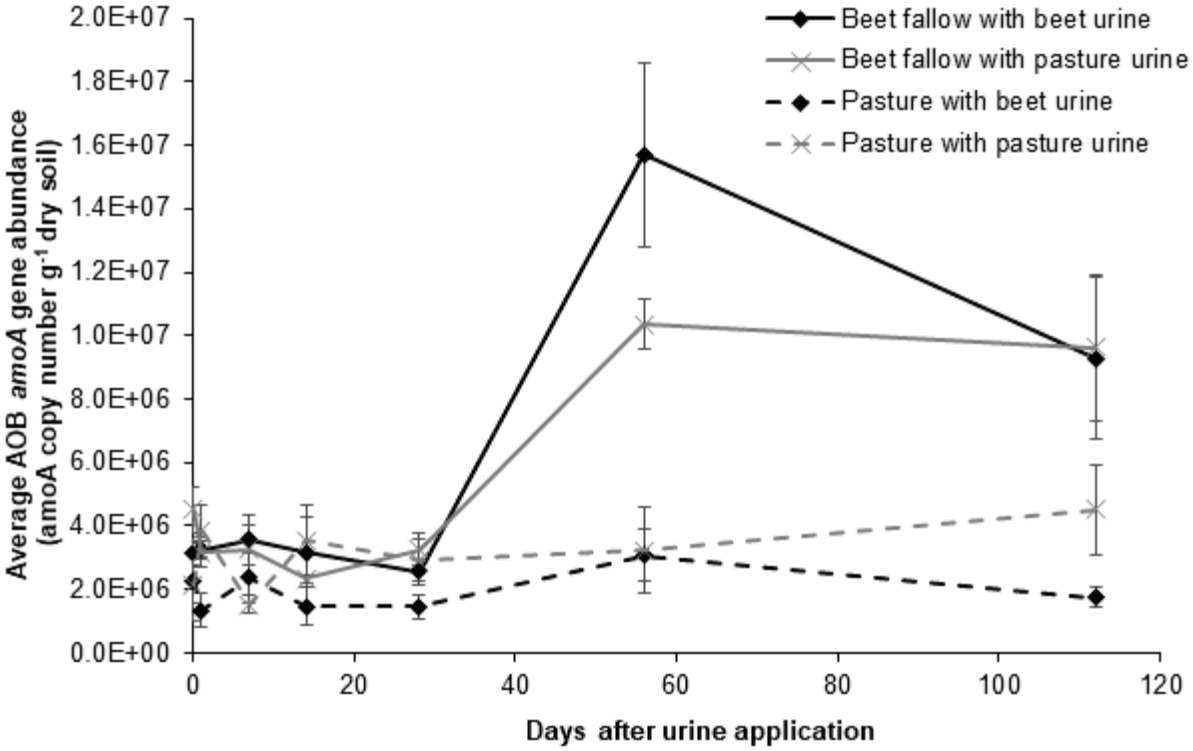


Figure F-1. The effect of urine type on soil ammonia oxidising bacteria (AOB) *amoA* gene copies over the first 112 days after urine application, beneath pasture. Day 0 is prior to urine application. Samples were taken from 0-100 mm depth in the soil profile. Error bars are standard error of the mean (*n* = 5).

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